REMARKS

INTERVIEW SUMMARY

On November 16, 2007, a telephonic Examiner's Interview was conducted between Jihong Zang, Applicants' attorney, and Examiner Chih Min Kam. We thank the Examiner for her participation in the Interview. During the Interview, all objections and rejections raised in the Office Action of July 9, 2008, were discussed. The Examiner also provided guidance with respect to overcoming the §112, first paragraph rejections.

AMENDMENT TO THE CLAIMS

Claim 23 has been amended to recite, "...(b) introducing a mutation causing a biotin auxotrophy into the biotin (*bio*) biosynthetic operon of the microorganism to control biomass production and which does not compromise the ability of the microorganism to produce said target fermentation product, and..." Support for this amendment may be found in the specification at, for example, page 12, lines 14-20; page 15, lines 9-21; page 17, lines 14-22; Examples 1-4 (pages 19-27); and figures 3 and 4.

Claim 24 has been amended to recite, "[t]he process according to claim 23 wherein step (b) comprises introducing a polynucleotide comprising a deletion-insertion mutation into the biotin (*bio*) biosynthetic operon of the microorganism to disrupt the microorganism's ability to produce biotin." Support for this amendment may be found in the specification at, for example, page 17, lines 14-22; Examples 1-4 (pages 19-27); and figures 3 and 4.

Claims 25-30 have been amended to recite "[t]he process according to..." rather than "[a] process according to..." Support for these amendments may be found in original claims 25-30. *See In re Gardner*, 177 USPQ 396, 397 (CCPA 1973) and MPEP §§ 608.01(o) and (l).

Claims 41-45 have been added. Support for claims 41-42 may be found in the specification at, for example, page 12, lines 14-20; page 15, lines 9-21; and figures 3 and 4. Support for claims 43-45 may be found in the specification at, for example, page 12, lines 14-20; page 15, lines 9-21; page 17, lines 14-22; Examples 1-4 (pages 19-27); and figures 3 and 4.

It is submitted that no new matter has been introduced by the foregoing amendments. Approval and entry of the amendments is respectfully solicited.

Objection

Claims 24-29 were objected to for containing an "informality." (Paper No. 20080701 at 2). With a view towards furthering prosecution, these claims have been amended to recite "[t]he process according to claim..." and, it is submitted, the objection is rendered moot and should be withdrawn.

Enablement Rejection

Claims 23-24 and 32 were rejected under 35 USC §112, first paragraph, on the asserted grounds that they contain subject matter which was not described in the

specification in such a way as to enable one skilled in the art to make and use the invention. (Paper No. 20080701 at 2-11).

In making the rejection, the Examiner asserted that the specification

> does not reasonably provide enablement for a process for decoupling production of a target fermentation product (i.e., riboflavin) from biomass production in a fermentation medium, the method comprising: (a) providing a recombinantly produced microorganism of bacillus that contains a polynucleotide sequence which encodes biosynthetic enzymes for the target fermentation product (i.e., riboflavin), (b) introducing a mutation causing biotin auxotrophy into the microorganism to control biomass production, and (c) supplying the medium with unlimited amount of substrates for producing the riboflavin and with a limited amount of biotin complementing the auxotrophy; and a microorganism made by the process, where the mutated gene causing biotin auxotrophy is not identified. (*Id.* at 3)

The Examiner, however, acknowledged that the specification is

> enabling for a process for decoupling production of a specific target fermentation product (i.e., riboflavin) from biomass production in a fermentation medium, the method comprising: (a) providing a recombinantly produced microorganism of bacillus that contains a polynucleotide sequence which encodes biosynthetic enzymes for the target fermentation product (i.e., riboflavin), (b) introducing a mutation causing a biotin auxotrophy into a specific gene of the microorganism such as bioFDB gene cassette (e.g., SEQ ID NO: 1) to control biomass production, and (c) supplying the medium with unlimited amount of substrates for producing the riboflavin and with a limited amount of biotin complementing the auxotrophy; and a microorganism made by the process. (*Id.*)

The asserted basis for the rejection appears to be the alleged breadth of step (b) in claim 23. In particular, the Examiner asserted:

> While the genes involved in biotin biosynthesis are known in the art, a convenient means may be used to introduce a mutation in the genes involved in biotin biosynthesis, and a screening method may be used to confirm a biotin auxotrophy, the claimed method recites the step (b) of introducing a mutation causing a biotin autotrophy into the microorganism to control biomass, in which the gene to be mutated is not identified, and the number of possible mutated genes to be tested is virtually endless. (*Id.* at 10-11).

The Examiner concluded, "the scope of the claim is broad, the working example does not demonstrate the claimed method associated with variants, the teachings in the specification are limited, and the identities of biotin auxotrophy-causing genes are unpredictable, and therefore, it is necessary to carry out undue experimentation to identify the mutated genes causing biotin auxotrophy." (*Id.* at 8).

Although not explicitly set forth in the statute, enablement may be found where some experimentation (even a considerable amount) is required, so long as the experimentation is not "undue." *Ex parte Forman*, 230 USPQ 546, 547 (BPAI 1986); *see also In re Colianni*, 195 USPQ 150, 153 (J. Miller concurring) (CCPA 1977); and *In re Rainer*, 146 USPQ 218, 220-221 (CCPA 1965). The Federal Circuit, adopting the analysis set forth in *Forman*, has enumerated several factors which may be considered in determining whether claims require that one skilled in the art perform undue experimentation in order to practice the claimed subject matter: breadth of the claims; predictability or unpredictability of the art; relative skill of those in the art; state of the prior art; nature of the invention; working examples; amount of guidance; and quantity of experimentation necessary. *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). These factors are merely illustrative, not mandatory; they provide a general framework

for analysis. *Enzo Biochem v. Calgene Inc.*, 52 USPQ2d 1129, 1136 (Fed. Cir. 1999); *Amgen, Inc. v. Chugai Pharm. Co.,* 18 USPQ2d 1016, 1027 (Fed. Cir., 1991), *cert. denied*, 502 U.S. 856 (1991).

In fact, enablement may still be present when an application contains no working examples or when prophetic examples are used. *Atlas Powder Co. v. E.I. du Pont De Nemours & Co.*, 224 USPQ 409, 414 (Fed. Cir. 1984) ("Use of prophetic examples, however, does not automatically make a patent non-enabling.") and *Strahilevitz*, 212 USPQ at 563 ("Nevertheless, as acknowledged by the board, examples are not required to satisfy section 112, first paragraph.").

With a view towards furthering prosecution, claims 23 and 24 have been amended to recite that the mutation is introduced "into the biotin (*bio*) biosynthetic operon." Thus, the gene to be mutated is identified, and "the number of possible mutated genes to be tested" is not endless. (Paper No. 20080701 at 11). Accordingly, as amended, it is respectfully submitted that a person skilled in this art would be able to practice the claimed subject matter without undue experimentation.

Indeed, as the Examiner acknowledged, the specification discloses working examples of biotin auxotrophs. In particular, examples 1-3 demonstrate the use of a BDF cassette to generate biotin auxotrophs and the use of such auxotrophs in the over-production of riboflavin. (Pages 19-27).

The specification also provides guidance as to the methods of creating auxotrophic mutants as well as the DNA sequences that may be targeted in order to create such biotin mutants. For example, FIG. 4 of the specification shows the structure

of the well-characterized biotin (*bio*) biosynthetic operon of *B. subtilis*, which comprises *bioW, bioA, bioF, bioD,* and *bioB*. The specification further discloses that mutations causing auxotrophic growth may be introduced using "any convenient means including, for example, chemical and UV mutagenesis, followed by screening or selection for a desired phenotype, construction of dysfunctional genes *in vitro* by recombinant techniques used to replace the intact counterparts of the genes in the genome of the microorganism..." (page 8, lines 17-21).

Mutated genes and other DNA sequences in the *bio* operon causing biotin auxotrophy are also well-known in the art. As the Examiner acknowledged, the related art discloses "the genes of the biotin biosynthetic operon of *Bacillus subtilis*; and insertion and deletion in the specific genes of [the] bio operon that cause biotin auxotrophy." (Paper No. 20080701 at 5). For example, as early as 1975, Pai *et al.*, "Genetics of Biotin Biosynthesis in *Bacillus subtilis*", J. Bacteriology, 121(1): 1-8 (1975) ("Pai", attached hereto as Exhibit A) disclosed the isolation of 11 independent biotin auxotrophs in *B. subtilis* by classic genetic methods of mutagenesis and followed by replica-plating techniques. (Pai, page 3, column 1, line 13 to page 4 column 2, line 24; *see* also Pai, page 3, Table 1). Six of the eleven auxotrophs had mutations in the *BioA* gene, and three had mutations in the *BioB* gene. (Pai, page 3, Table 1). Later works confirmed and extended Pai's disclosure. For example, Bower *et al.*, "Cloning, Sequencing, and Characterization of the *Bacillus subtilis* Biotin Biosynthetic Operon," J. Bacteriology 178: 4122-4130 (1996) ("Bower", attached hereto as Exhibit B) disclosed specific regions of the bio operon that causes biotin auxotrophy. Bower discloses that

the insertion of a *cat* gene in *bioW* (mutant C, Ω*bioW*) and deletion in *bioB* (mutant F, Δ*bioB*) causes biotin auxotrophy in *B. subtilis*. (*See* Bower, Table 4, page 4128). Bower's disclosure that the gene product of *BioW* is required for biotin synthesis was also confirmed by the disclosure of Sasaki *et al.*, "Genetic Analysis of an Incomplete *bio* Operon in a Biotin Auxotrophic Strain of *Bacillus subtilis* Natto OK2," Biosci. Biotechnol. Biochem., 68(3): 739-742 (2004) ("Sasaki", attached hereto as Exhibit C), which disclosed that mutations in the *bioW* gene resulted in biotin auxotrophy.

In fact, one skilled in this art understands that not only the genes but also the promoter sequences in the *bio* operon are important for biotin synthesis in *B. subtilis*. For example, Bower disclosed that "replacement of the region upstream of *bioW* containing the putative P_{bio} promoter with the *cat* gene oriented opposite to the biotin operon ... generated an unambiguous Bio^- phenotype." (Bower, page 4127, column 2, lines 11-15; *see* also Bower, Table 4, page 4128, mutant G, ΔP_{bio}).

The level of knowledge and skill in this art is high. (Declaration of Dr. Nigel J. Mouncey, previously submitted with the Response mailed on February 7, 2008, a copy of which is attached hereto as Exhibit D, ¶15). Given the disclosure in the art and the high level of skill in the art, one skilled in the art could easily generate biotin auxotrophs by disrupting the function of the genes and sequences in the *bio* operon. For example, one skilled in this art may manipulate the DNA sequences in the *bio* operon, which was submitted to GenBank under accession number U51868. (Bower, page 4124, column 1, lines 7-9). Furthermore, as demonstrated by Bower, these manipulations may include insertion or deletion of exogenous DNA sequences into the

genes or promoter sequences of the *bio* operon. Thus, such manipulations were well within the skill of the art at the time of the application. Similarly, genetic manipulations may also include introduction of point mutations to create stop codons in the genes of the *bio* operon to create dysfunctional genes. Even the Examiner conceded that "a convenient means may be used to introduce a mutation in the genes involved in biotin biosynthesis." (Paper No. 20080701 at 10).

Thus, creating a biotin auxotroph is very predictable. Disrupting the function of genes and sequences of the biotin operon was easily achievable by one skilled in the art, as outlined above. Furthermore, as Dr. Mouncey pointed out, it was well within the skill of the art to generate and screen for biotin auxotrophic mutants and that such work was routine and well within the skill of the art. (Declaration, ¶ 17). Dr. Mouncey's Declaration is further supported by Pai, which demonstrates that as early as 1975, 11 biotin auxotrophs were generated and found by screen.

Given the specification's disclosure of the methods of generation of biotin auxotrophic mutants, the actual working example of an auxotrophy, the detailed knowledge of one skilled in the art of sequence and function of various essential sequences in the *bio* operon and how to make biotin auxotrophs, the high level of skill in the art, and the predictability of generation of a biotin auxotroph, it is respectfully submitted that the claims, as amended, are enabled. Thus, for the reasons set forth above, the rejection should be withdrawn.

Written Description Rejection

Claims 23-24 and 32 were rejected under 35 USC §112, first paragraph, as containing subject matter that was not described in the specification in such a way to convey that the inventors, at the time the application was filed, had possession of the claimed invention. (Paper No. 20080701 at 11-15).

In making the rejection, the Examiner asserted that

> [w]hile the specification indicates that the invention provides a process for decoupling production of a target fermentation product from biomass production in a fermentation medium by introducing a specific biotin auxotroph mutant construct comprising SEQ ID NO: 1 into *bacillus subtilis* RB50 containing multiple copies of the engineered *rib* operon pRF69, culturing fermentations, and measuring biomass and riboflavin production at different biotin concentrations, which shows the product yield (i.e., the amount of riboflavin produced on the consumed glucose) is 33% higher in the decoupled process to the coupled process (see Examples 1-3), the specification does not disclose a genus of variants for mutated genes that cause biotin auxotrophy in a transformed microorganism as encompassed by the claims. (*Id.* at 12-13).

The Examiner further asserted that "[i]ntroducing a single species of a mutated gene ...into the microorganism... does not provide written description for the genus of variants of mutated genes that cause biotin auxotrophy in the claimed method, which would encompass identifying mutated genes causing biotin auxotrophy from numerous mutated genes." (*Id.* at 13) The Examiner also asserted, "without guidance on the structures of various mutated genes that cause biotin auxotrophy, one skilled in the art would not know the identities of the mutated genes that cause biotin auxotrophy." (*Id.*) The Examiner then concluded, "[t]he lack of description on the structures of the mutated genes that cause biotin auxotrophy, and the lack of representative species as

encompassed by the claims, applicants have failed to sufficiently describe the claimed invention, in such full, clear, concise terms that a skilled artisan would not recognize applicants were in possession of the claimed invention." (*Id.*) The Examiner further asserted that the previous response and Declaration of Dr. Nigel J. Mouncey was unpersuasive because allegedly, "numerous mutated genes can be introduced into microorganism, and there is no structure to function/activity correlation established for the mutated genes, a skilled person would not know how to choose a proper mutated gene[] that cause biotin auxotrophy other than the specific mutated genes in bio operon as indicated in Bower." (*Id.* at 15).

As noted above, with a view towards furthering prosecution, claims 23 and 24 have been amended to recite that the mutation is introduced "into the biotin (*bio*) biosynthetic operon." Thus, "the identities of the mutated genes that cause biotin auxotrophy" are clearly defined in the amended claim. (Paper No. 20080701 at 13). Furthermore, as set forth above, the art at the time the instant application was filed disclosed that mutated genes in the *bio* operon cause biotin auxotrophy, and generation of biotin auxotrophs was predictable and well within the skill of the artisan. Therefore, one skilled in this art would know "how to choose a proper mutated gene[] that cause[s] biotin auxotrophy". (Paper No. 20080701 at 15) Accordingly, it is respectfully submitted that the Applicants were in possession of the full scope of the instantly claimed invention at the time the application was filed and that the rejection should be withdrawn.

Thus, for the reasons set forth above, entry of the amendments, withdrawal of the objection and rejections, and allowance of the claims are respectfully requested. If the Examiner has any questions regarding this paper, please contact the undersigned.

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on January 8, 2009.

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Genetics of Biotin Biosynthesis in *Bacillus subtilis*

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Biotin auxotrophs of *Bacillus subtilis* were isolated and classified into three groups according to growth requirements, cross-feeding pattern, and biotin precursors excreted into culture supernatant fluids. Mutant genes were mapped by transduction using phage PBS1. All presently identified *bio* genes were linked to *aroG* with an order of *bio-aroG-argA-leu-1*. No linked markers were found to the left of the *bio* loci.

The pathway for biotin biosynthesis as it has been shown in *Escherichia coli* is shown in Fig. 1 (4, 6, 8, 9, 16, 18). The genetic loci that determine the structure of the enzymes involved in the pathway are clustered at min 17.5 on the genetic map (5, 6, 23) and constitute two divergently transcribed operons (5, 10). Reactions that lead to the formation of pimelyl-coenzyme A are not known, although two genes, *bioC* in the *bio* cluster (6, 23) and *bioH* at min 66 on the genetic map (11), have been implicated in undetermined steps prior to pimelyl-coenzyme A. Feeding studies with mutants blocked prior to 7-oxo-8-aminopelargonic acid (7-KAP) are not possible because *E. coli* is impermeable to pimelic acid (22).

In this study, the genetic and biochemical aspects of the biotin pathway in *Bacillus subtilis* have been examined for the purpose of comparison with the *E. coli* system. *B. subtilis* was chosen because of the availability of a suitable genetic system and its permeability to pimelic acid (C. H. Pai, unpublished data).

MATERIALS AND METHODS

Bacterial strains. The strains of *B. subtilis* and *E. coli* used are listed in Table 1. The strains were obtained through J. Hoch of the Scripps Clinic and Research Foundation from C. Anagnostopoulos, E. Nester, and B. Reilly. All *B. subtilis* strains were stored frozen in a medium containing (per liter) neopeptone (10 g) and glycerol (100 ml).

Media and cultural conditions. The minimal medium of Spizizen (25) was used. When required, the minimal medium was supplemented aseptically with L-amino acids (25 μg/ml), adenine (100 μg/ml), uracil (100 μg/ml), or biotin (5 ng/ml). For Casamino Acids Medium, the minimal medium was supplemented with Difco vitamin-free Casamino Acids (2 g/liter). Two nutrient media were used: Difco antibiotic medium 3 (Penassay) and Difco tryptose blood agar base. All liquid cultures were incubated at 37 C with vigorous shaking.

Identification of biotin precursors from culture supernatant fluids. Cells grown overnight in the minimal medium supplemented with pimelic acid (100 μg/ml) and biotin (0.5 ng/ml) were centrifuged, and the supernatant fluids were chromatographed with a solvent system of *n*-butanol-acetic acid-water (4:1:5). Spots for biotin precursors, desthiobiotin (DTB), 7,8-diaminopelargonic acid (DAP), and 7-KAP, were identified by bioautographic techniques described previously (19, 20).

Biotin cross-feeding. The ability of *bio* mutants of *B. subtilis* to cross-feed other biotin auxotrophs was examined using the four *bio* strains of *E. coli* that are listed in Table 1. *E. coli* strains K12-20, K12-1, K12-26, and K12-22 have the following biosynthetic blocks:

$$\xrightarrow{\text{K12-22}} \text{7-KAP} \xrightarrow{\text{K12-26}} \text{DAP} \xrightarrow{\text{K12-1}}$$

$$\text{DTB} \xrightarrow{\text{K12-20}} \text{biotin}$$

Cross-feeding plates were prepared with Casamino Acids medium containing 2,3,5-triphenyltetrazolium chloride (50 mg/liter) and a washed-cell suspension of one of the four *E. coli* indicator strains. Heavy suspensions of test strains were streaked on the cross-feeding plates, which were then incubated at 37 C for 2 days. Since the medium did not contain biotin, the growth of test organisms was limited but was enough to cross-feed the indicator strains. Cross-feeding was scored as positive when growth of seeded cells was indicated by the appearance of pink colonies under and around the streak.

Propagation of bacteriophage. A fresh culture of *Bacillus licheniformis* 8480 was infected with phage PBS1 and plated in soft agar (tryptone, 1%; NaCl, 0.8%; glucose, 0.6%; agar, 0.6%) onto fresh tryptose blood agar base plates. A single plaque was picked and the above procedure was repeated. After 10 h of incubation at 37 C, the soft-agar layer was scraped and the surface of the plate was washed with 2 ml of broth into a centrifuge tube. Supernatant fluids were filtered through membrane filters (Millipore Corp.).

Transduction. Transducing lysates of phage PBS1 were prepared by the procedures of Young et al. (27).

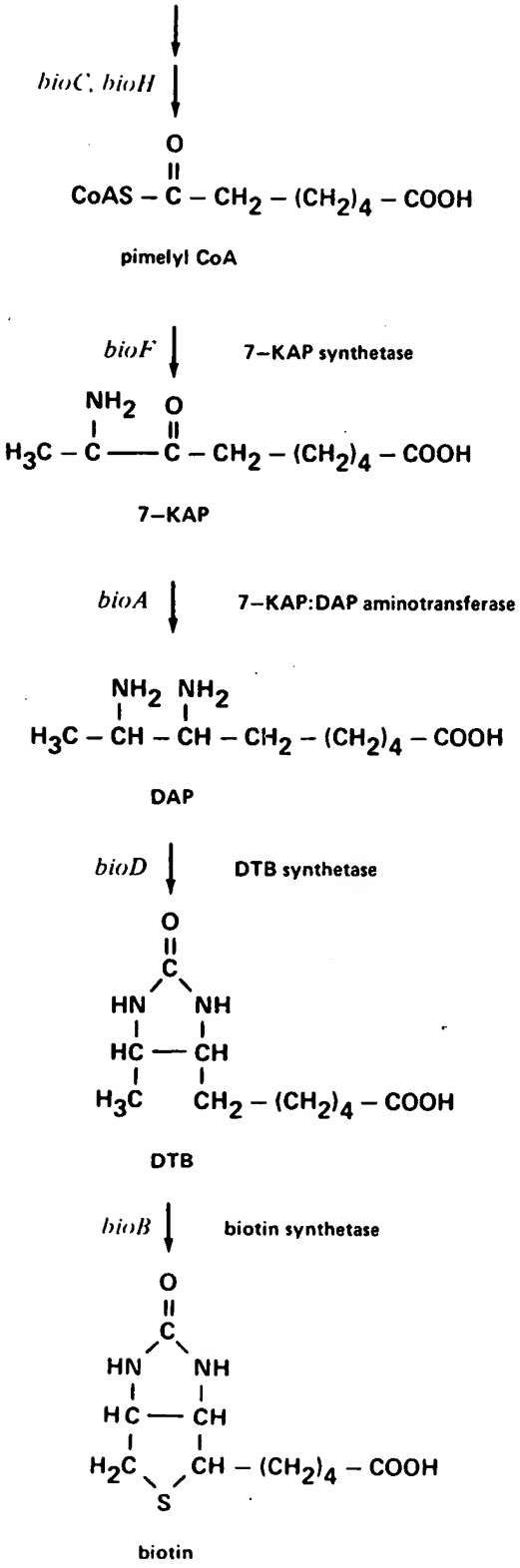

FIG. 1. *Biotin biosynthetic pathway in Escherichia coli.*

Two of the original *bio* mutants were nonmotile, and motile revertants were isolated by streaking on tryptose blood agar base plates containing 0.7% agar. When a swarm was observed on the plates, cells from the edge of the swarm were restreaked on normal tryptose blood agar base plates to obtain pure cultures of motile revertants.

For transduction, single colonies of recipient strains grown overnight at 37 C on tryptose blood agar base plates were inoculated into a tube containing 5 ml of Penassay broth. The cultures were incubated for 3 to 5 h with vigorous shaking until cells became fully motile, and were infected with phage PBS1 lysates at a multiplicity of infection of 1.0. The infected cultures were incubated for 15 min and centrifuged to remove supernatant fluids. The pellets were suspended in 2.0 ml of minimal salts (minimal medium minus glucose), and samples (0.05 to 0.1 ml) were plated onto selection medium containing appropriate supplements. The plates were incubated for 2 to 4 days, and only those plates with more than 50 transductants were examined for genetic analyses.

Transformation. Transforming deoxyribonucleic acid was prepared by the method of Young and Spizizen (28), except that Pronase was used instead of trypsin and deoxyribonucleic acid was extracted by phenol. For phenol extraction, an equal volume of phenol [saturated with tris(hydroxymethyl)aminomethane-hydrochloride, 10 mM, pH 7.5] was added to cell lysate, and the mixture was shaken for 10 min. The phenol layer was carefully removed after centrifugation, dialyzed against saline solution (0.8%) for 8 h, and then dialyzed against a solution containing sodium chloride (0.87%) and sodium citrate (0.441%) for 8 h.

Preparation of competent cells and transformation were by the methods of Anagnostopoulos and Spizizen (2). When strains carrying *aroG* marker were used as recipients, media were supplemented with Difco nutrient broth (dehydrated) at 4 g and 0.4 g per liter in growth and transformation media, respectively. The addition of nutrient broth stimulated the formation of competence in *aroG* strains (C. H. Pai, unpublished data).

Mitomycin susceptibility. Mitomycin C susceptibility, which was used as an indicator for the presence of *recA* marker (13), was tested on minimal agar containing required growth factors and mitomycin C (0.05 μg/ml). The wild-type strains are fully resistant to the above concentration of the antibiotic, whereas *recA* strains show no growth after 24 h of incubation. Mitomycin plates were stored in the dark.

Scoring of polA marker. Deoxyribonucleic acid polymerase I-deficient mutants (*polA*) are sensitive to methyl methane sulfonate (17). Methyl methane sulfonate sensitivity was tested on a minimal agar containing required supplements and 0.047% methyl methane sulfonate. The agar medium was cooled to 45 C, and the methyl methane sulfonate solution was added aseptically just before the medium was poured. Sensitivity was scored after 16 h of incubation at 37 C. Because of the instability of methyl methane sulfo-

TABLE 1. *List of bacterial strains*

Strain		Genotype[a]	Origin or reference
B. subtilis 168		*trpC2*	From stock
	BR13	*trpC2 pyrA*	B. Reilly
	GSY384	*argA11 leu-1*	C. Anagnostopoulos
	GSY1025	*trpC2 metB4 recA1*	C. Anagnostopoulos
	GSY1070	*trpC2 pheA*	C. Anagnostopoulos
	NCIB[b] 10265	*trpC2 tyrA*	NCIB
	SB5	*trpC2 hisA1 pyrA*	E. Nester
	SB19	*str-1*	E. Nester
	SB1063	*polA5 pheA*	Laipis and Ganesan (17)
	WB932	*aroG932*	Hoch and Nester (15)
	C50	*trpC aspA*[c]	J. Hoch
	JKB112	*bio-112 str-1*	Mutagenesis of SB19
	JKB141	*bioB141 str-1*	Mutagenesis of SB19
	JKB152	*bioB152 str-1*	Mutagenesis of SB19
	JKB173	*bioA173 str-1*	Mutagenesis of SB19
	JKB181	*bioA181 str-1*	Mutagenesis of SB19
	JKB214	*bioB214 trpC2 tyrA*	Mutagenesis of NCIB 10265
	JKB216	*bioA216 trpC2 tyrA*	Mutagenesis of NCIB 10265
	JKB222	*bioA222 trpC2 tyrA*	Mutagenesis of NCIB 10265
	JKB223	*bioA223 trpC2 tyrA*	Mutagenesis of NCIB 10265
	JKB231	*bioA231 trpC2 tyrA*	Mutagenesis of NCIB 10265
	JKB235	*bio-235 trpC2 tyrA*	Mutagenesis of NCIB 10265
	JKB504	*bioB141 argA11*	Transduction GSY384 by PBS1 grown on JKB141, selection for Leu$^+$
	JKB1141	*bioB141 leu-1*	Transduction GSY384 by PBS1 grown on JKB141, selection for Arg$^+$
	JKB3141	*bioB141 aroG932*	Transduction JKB504 by PBS1 grown on WB932, selection for Arg$^+$
E. coli	K12-20	*bioB20*	Mutagenesis of K-12 (18, 23) lacks biotin synthetase activity
	K12-1	*bioD1*	Mutagenesis of K-12 (18, 19) lacks DTB synthetase activity
	K12-26	*bioA26*	Mutagenesis of K-12 (18, 20) lacks 7-KAP:DAP aminotransferase activity
	K12-22	*bio-22*	Mutagenesis of K-12 (18) blocked prior to 7-KAP

[a] The genetic symbols for *B. subtilis* are as described in Young and Wilson (29), and those for *E. coli* are as described by Taylor and Trotter (26).

[b] National Collection of Industrial Bacteria, Aberdeen, Scotland.

[c] Mutants in the *aspA* locus are devoid of pyruvate carboxylase and respond to either glutamate or aspartate (14).

nate, plates were stored at 4 C and discarded after a week.

Chemicals. Mitomycin C and methyl methane sulfonate were purchased from Sigma Chemical Co., St. Louis, Mo., and Eastman Kodak Co., Rochester, N.Y., respectively. DAP was a generous gift from Hoffman-La Roche Inc., Nutley, N.J., and 7-KAP acid was prepared by the method of Suyama and Kaneo (Japanese patent no. 197160). All other chemicals were of reagent grade obtained commercially.

RESULTS AND DISCUSSION

Isolation of bio mutants. *B. subtilis* strains SB19 and NCIB 10265 were mutagenized with nitrosoguanidine (1) and plated onto Casamino Acids agar supplemented with biotin (5 ng/ml). Eleven independent biotin auxotrophs were isolated by replica-plating techniques.

Characterization of mutants. Preliminary characterization of these mutants indicated that the most of them had acquired additional mutations with respect to growth requirements, colonial morphology, motility, or sporulation. They exhibited a considerable difference in the amount of growth in liquid media. It was therefore desirable to obtain isogenic strains carrying each mutant allele to examine their biochemical properties. Since all mutant genes were co-transducible with *argA* (see the results of genetic studies), PBS1 lysates prepared on each of *bio* mutants were used to transfer *bio* markers into strain GSY 384 (*argA leu-1*) select-

ing for Arg$^+$. Arg$^+$Bio$^-$Leu$^-$ strains were analyzed for the following properties: (i) ability to grow on biotin, DTB, DAP, 7-KAP, or pimelic acid; (ii) ability to cross-feed four *bio* strains of *E. coli* that are blocked at different steps of the biotin pathway; and (iii) excretion of biotin precursors in culture supernatant fluids.

From the results shown in Table 2, the mutants were classified into three groups. The mutants in group I grew on biotin only with an excretion of DTB and 7-KAP. They could cross-feed strains K12-1, K12-26, and K12-22 of *E. coli*. The *bioB* mutants of *E. coli*, which lack biotin synthetase activity, have the same properties as those of group I mutants (18, 24). The bioautographic techniques used to identify biotin precursors in culture supernatant fluids are not sensitive enough to detect DAP because of extremely low growth-promoting activity (18, 20). Group II included those mutants that could grow on either biotin, DTB, or DAP, but not on 7-KAP. These mutant strains excreted 7-KAP in culture supernatant fluids and cross-fed *E. coli* strain K12-22 only. These are the characteristics of *E. coli* with a mutation in the *bioA* gene that codes for 7-KAP:DAP aminotransferase (18, 24). Those mutant strains classified into group III grew on all of the compounds tested except pimelic acid. They did not cross-feed any of the *E. coli* strains, and no biotin precursors were accumulated in culture supernatant fluids. These results suggested that group III mutants were blocked at steps prior to 7-KAP. The finding that the amounts of biotin precursors accumulated in culture supernatant fluids of groups I and II mutants were much larger when cells were grown in the presence of pimelic acid (100 μg/ml) (C. H. Pai, unpublished data) suggested that pimelic acid was utilized as a biotin precursor in *B. subtilis*. However, none of the mutants was able to grow on pimelic acid.

Using the gene designation employed in *E. coli*, the mutant genes in groups I and II were termed *bioB* and *bioA*, respectively. Group III mutants could have defects in any one or more of *bio* genes that code for enzymes involved in the formation of 7-KAP and, therefore, were designated simply *bio-112* and *bio-235*. The possibility exists, however, that these mutants were blocked at the 7-KAP synthetase step since they did not grow on pimelic acid. 7-KAP synthetase mutants in *E. coli* are designated *bioF*. No mutants were found that possessed the characteristics of the *bioD* mutation in *E. coli*. The *bioD* gene specifies DTB synthetase that catalyses the conversion of DAP to DTB.

Mapping of bioB locus. PBS1 lysates prepared on strain JKB141 (*bioB141*) were used to transduce a number of auxotrophic markers that are roughly evenly distributed on the *B. subtilis* chromosome according to the linkage map of Young and Wilson (29; Fig. 2). Of *purA*, *purB*, *thr-1*, *cys-B*, *hisA*, *argC*, *metC*, *pyrA*, *argA*, *pheA*, *aroD*, *lys*, *trpC*, *metB*, and *gap*

TABLE 2. *Properties of B. subtilis bio mutants*

bio allele	Growth[a]					Cross-feeding[b]				Excretion of biotin[c] precursors in culture supernatant fluids
	B	DTB	DAP	7-KAP	P	*E. coli* K12-20	*E. coli* K12-1	*E. coli* K12-26	*E. coli* K12-22	
141	+	−	−	−	−	−	+	+	+	DTB and 7-KAP
152	+	−	−	−	−	−	+	+	+	DTB and 7-KAP
214	+	−	−	−	−	−	+	+	+	DTB and 7-KAP
173	+	+	+	−	−	−	−	−	+	7-KAP
181	+	+	+	−	−	−	−	−	+	7-KAP
216	+	+	+	−	−	−	−	−	+	7-KAP
222	+	+	+	−	−	−	−	−	+	7-KAP
223	+	+	+	−	−	−	−	−	+	7-KAP
231	+	+	+	−	−	−	−	−	+	7-KAP
112	+	+	+	+	−	−	−	−	−	None
235	+	+	+	+	−	−	−	−	−	None

[a] Abbreviations: B, *d*-biotin; P, pimelic acid. Minimal agar supplemented with L-leucine (25 μg/ml) was used. Concentrations of biotin and biotin precursors used were: biotin, 5 ng/ml; DTB, 10 ng/ml; DAP 270 ng/ml; 7-KAP, 1.2 μg/ml; P, (100 μg/ml). +, Growth; −, no growth.

[b] Cross-feeding plates seeded with indicator strains of *E. coli* were used. +, Positive cross-feeding; −, negative cross-feeding.

[c] Cells were grown in minimal medium supplemented with L-leucine (25 μg/ml), pimelic acid (100 μg/ml), and a suboptimal concentration of biotin (0.5 ng/ml). Culture supernatant fluids were chromatographed, and biotin precursors were identified by bioautographic techniques.

markers that were examined, only *argA* was found to be linked to *bioB* by transduction. The location of the *bioB* was examined in detail by determining recombination frequencies with *pheA*, *leu-1*, *argA*, *aroG*, and *pyrA* (Table 3). The *bioB* marker was strongly linked to *aroG932*, which was previously shown to be located on the left of *argA* (15). The *bioB* gene was not co-transducible with *pyrA*. A linkage gap between *pyrA* and *argA* (7) or between *pyrA* and *aroG* (15) was also found by other investigators. The recombination values obtained by the two-factor crosses suggested a linear order for *bioB*, *aroG*, *argA*, and *leu-1*.

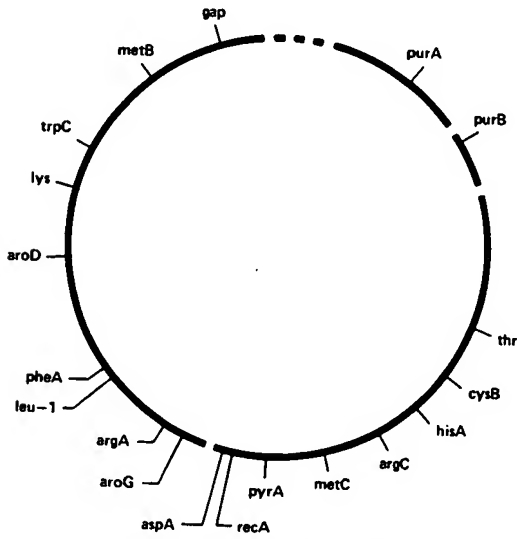

FIG. 2. *Linkage map of Bacillus subtilis. The location and symbols of the markers used in this study are according to Young and Wilson (29).*

It was noted during these studies that Arg$^-$ colonies did not grow as well as Arg$^+$ on medium containing L-arginine (25 μg/ml). For example, in a cross between an Arg$^-$ Leu$^-$ strain (recipient) and a Bio$^-$ strain (donor) selecting for Leu$^+$, there were two types of colonies, large and small, after 2 days of incubation. The large colonies were almost entirely Arg$^+$, whereas small ones were Arg$^-$. Upon further incubation (2 more days), the difference in the size of these two types of colonies became less obvious. A similar observation was made by Young et al. (27) when an *argC* marker was used in transduction. Therefore, in all subsequent experiments in which *argA* was used as an unselected marker, plates were incubated for 4 days before transductants were scored for their phenotype.

The orientation of *bioB* with respect to *aroG*, *argA*, and *leu-1* markers was established by three-factor transduction crosses shown in Table 4. The results were consistent with the order *bioB-aroG-argA-leu-1*, which agreed well with the one suggested by the two-factor crosses.

Mapping of other bio loci. Having established the location of *bioB141*, we examined the

TABLE 3. *Mapping of bioB141 by two-factor crosses by PBS1 transduction*

Donor and genotype	Recipient and genotype	Selection	Phenotype of recombinants	No.	Recombination[a] (%)
JKB141 (*bioB141*)	GSY1070 (*pheA*)	Phe$^+$	Bio$^+$	208	100
			Bio$^-$	1	
JKB141 (*bioB141*)	GSY384 (*argA11 leu-1*)	Leu$^+$	Bio$^+$	316	90
			Bio$^-$	34	
168 (*bio$^+$ leu$^+$*)	JKB1141 (*bioB141 leu-1*)	Leu$^+$	Bio$^+$	32	91
			Bio$^-$	340	
JKB141 (*bioB141*)	GSY384 (*argA11 leu-1*)	Arg$^+$	Bio$^+$	145	59
			Bio$^-$	104	
168 (*bio$^+$ arg$^+$*)	JKB504 (*bioB141 argA11*)	Arg$^+$	Bio$^+$	86	59
			Bio$^-$	122	
JKB3141 (*bioB141 aroG932*)	GSY384 (*argA11 leu-1*)	Arg$^+$	Bio$^+$	85	61
			Bio$^-$	53	
JKB141 (*bioB141*)	WB932 (*aroG932*)	Aro$^+$	Bio$^+$	71	36
			Bio$^-$	128	
168 (*bio$^+$ aro$^+$*)	JKB3141 (*bioB141 aroG932*)	Aro$^+$	Bio$^+$	122	35
			Bio$^-$	65	
JKB141 (*bioB141*)	BR13 (*pyrA*)	Ura$^+$	Bio$^+$	231	100
			Bio$^-$	0	
JKB141 (*bioB141*)	SB5 (*pyrA*)	Ura$^+$	Bio$^+$	178	100
			Bio$^-$	0	

[a] 100 – percent co-transduction.

TABLE 4. *Mapping of bioB141 by three-factor transduction crosses*

Cross	Markers[a]			No. of recombinants	Order implied by results
	bio	*arg*	*leu*		
Donor: *bioB141* Recipient: *argA11 leu-1*	1	1	1	34	*bioB-argA-leu-1*
	0	1	1	93	
	0	0	1	221	
	1	0	1	2	
	bio	*aro*	*leu*		
Donor: *aroG932* Recipient: *bioB141 leu-1*	1	1	1	38	*bioB-aroG-leu-1*
	0	1	1	38	
	0	0	1	188	
	1	0	1	1	
	bio	*aro*	*leu*		
Donor: *bioB141 aroG932* Recipient: *argA11*	1	1	1	53	*bioB-aroG-argA*
	0	1	1	36	
	0	0	1	71	
	1	0	1	0	

[a] The designations 1 and 0 refer to donor and recipient genotypes, respectively.

linkage relationship of other *bio* mutations with respect to *aroG* and *argA*. PBS1 lysates prepared on each of the *bio* mutants were used to transduce *aroG932* and *argA11* markers. The mutant genes of all classes of presently isolated biotin auxotrophs were linked to *aroG* and *argA* (Table 5). Furthermore, the frequency of recombination of all *bio* mutations with these two markers was approximately the same as that found for *bioB141*, suggesting that these *bio* loci were linked to each other and formed a cluster. A summary of genetic studies of the *bio* loci is shown in Fig. 3.

Mapping of bio loci by transformation. Transduction with PBS1 is a valuable tool for establishing linkages between distant markers, because of the large size of donor fragment in transducing particles (3, 7). Transformation, on the other hand, involves much smaller donor deoxyribonucleic acid (7) and is, therefore, useful for mapping very closely linked markers that recombine too infrequently to be easily mapped with phage PBSI.

To map the *bio* genes with respect to each other by three-factor transformation crosses, a closely linked outside marker must be available. Although the *aroG* was co-transduced with the *bio* markers at a frequency of about 65%, co-transformation frequency was found to be only 5% (Table 6). All three markers, *bioA173*, *bioB141*, and *bio-112*, representing each of three mutant classes were weakly linked to *aroG* by transformation.

Search for a possible reference marker for mapping of bio loci. Since *aroG932* was found to be too far removed from the *bio* loci, attempts were made to find a marker close enough to the *bio* loci to be used as a reference marker. Three markers, *recA* (12), *polA* (17), and *aspA* (14), have been reported which might be located very close to the *bio* loci.

Both *recA* and *aspA*, which are closely linked to each other, have been previously shown to be located on the right of *pyrA* and weakly linked to *argA* (12, 14). However, a linkage relationship of *recA* or *aspA* with *bio* or *aroG* could not be demonstrated in the present study (data not shown). Dubnau et al. (7) have shown four linkage groups of the *B. subtilis* chromosome that can not be ordered relative to one another.

TABLE 5. *Two-factor crosses of bio loci with aroG932 and argA11*

Donor	% Recombination[a] with:	
	aroG932 (WB932)	*argA11* (GSY384)
bioB141	35	49
bioB152	29	52
bioB214	43	51
bioA173	31	45
bioA181	36	46
bioA223	36	45
bioA231	37	62
bioA216	38	55
bioA222	32	50
bio-112	33	45
bio-235	31	43

[a] 100 − percent co-transduction.

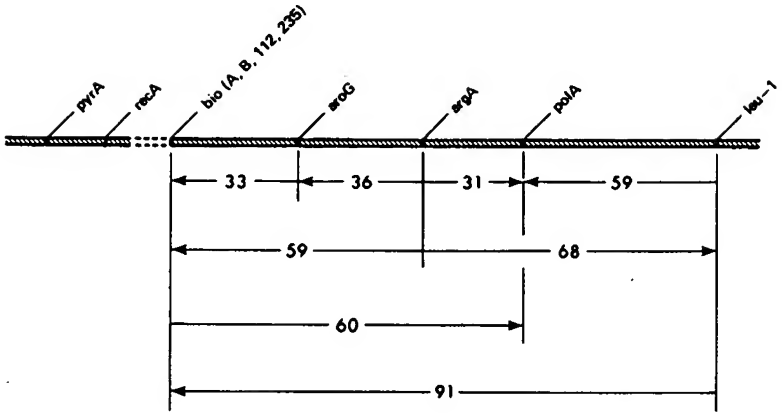

FIG. 3. *Genetic map showing the bio loci relative to other closely linked genes on the B. subtilis chromosome. The figures represent percent recombination by PBS1 transduction, and the arrowheads indicate the unselected markers. The bio markers have not been ordered with respect to each other. The position of pyrA and recA, which are unlinked to the bio loci, are according to Young and Wilson (29).*

TABLE 6. *Co-transfer of bio and aroG by transformation*[a]

Donor	Aro⁺Bio⁻/Aro⁺	Co-transfer (%)
bioA173	23/457	5
bioB141	39/769	6
bio-112	19/380	5

[a] Recipient strain, WB932 (*aroG932*); deoxyribonucleic acid concentration, 0.05 μg/ml.

The *bio* markers appear to be located at the one end of the linkage group III.

The *polA* marker was found to be located to the left of *aroG* and unlinked to *pyrA* (17), suggesting a possible tight linkage of the *polA* to the *bio*. The results of three-factor transduction analyses involving *bioB*, *aroG*, *polA*, *argA*, and *leu-1* (data not shown) did not agree with the previous finding and were suggestive of the order *bioB-aroG-argA-polA-leu-1*.

Search for a marker that is tightly linked to the *bio* locus may be facilitated by a technique of localized mutagenesis using nitrous acid-treated deoxyribonucleic acid (3).

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Cloning, Sequencing, and Characterization of the *Bacillus subtilis* Biotin Biosynthetic Operon

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A 10-kb region of the *Bacillus subtilis* genome that contains genes involved in biotin biosynthesis was cloned and sequenced. DNA sequence analysis indicated that *B. subtilis* contains homologs of the *Escherichia coli* and *Bacillus sphaericus bioA*, *bioB*, *bioD*, and *bioF* genes. These four genes and a homolog of the *B. sphaericus bioW* gene are arranged in a single operon in the order *bioWAFDB* and are followed by two additional genes, *bioI* and *orf2*. *bioI* and *orf2* show no similarity to any other known biotin biosynthetic genes. The *bioI* gene encodes a protein with similarity to cytochrome P-450s and was able to complement mutations in either *bioC* or *bioH* of *E. coli*. Mutations in *bioI* caused *B. subtilis* to grow poorly in the absence of biotin. The bradytroph phenotype of *bioI* mutants was overcome by pimelic acid, suggesting that the product of *bioI* functions at a step prior to pimelic acid synthesis. The *B. subtilis bio* operon is preceded by a putative vegetative promoter sequence and contains just downstream a region of dyad symmetry with homology to the *bio* regulatory region of *B. sphaericus*. Analysis of a *bioW-lacZ* translational fusion indicated that expression of the biotin operon is regulated by biotin and the *B. subtilis birA* gene.

Biotin biosynthesis in *Escherichia coli* and *Bacillus sphaericus* has been studied extensively at both the biochemical and molecular biological levels (9, 14, 17, 29). The enzymes involved in the conversion of pimeloyl coenzyme A (CoA) to biotin have been isolated from both of these bacterial species and characterized (2, 14, 16, 23, 29, 42). The analogous pairs of enzymes from the two species are similar, although some of the components involved in the last step in biotin synthesis remain to be elucidated (6, 15, 25, 26, 37, 46). 8-Amino-7-ketopelargonic acid (KAPA) synthase, the product of *bioF*, catalyzes the conversion of pimeloyl-CoA and alanine to KAPA (Fig. 1). 7,8-Diaminopelargonic acid (DAPA) aminotransferase, the product of *bioA*, then uses *S*-adenosylmethionine as a donor to transfer an amino group to KAPA, yielding DAPA. Dethiobiotin (DTB) synthetase (*bioD*) catalyzes the closure of the ureido ring to produce DTB, and finally the product of *bioB*, biotin synthase, functions together with a number of other components, including flavodoxin (6, 26), *S*-adenosylmethionine (6, 15, 25, 37, 46), and possibly cysteine (6, 15, 47), to convert DTB to biotin.

In *E. coli* the genes that encode these enzymes are located in two divergently transcribed operons, controlled by a single operator that interacts with the BirA repressor (1, 9). In *B. sphaericus*, the genes are located in two separate, unlinked operons (17). The early steps of the pathway, those involved in the synthesis of pimeloyl-CoA, are less well understood (27, 48). *B. sphaericus* contains an enzyme, pimeloyl-CoA synthetase (*bioW*), that converts pimelic acid to pimeloyl-CoA (17, 43). *E. coli* lacks this enzyme and cannot use pimelic acid as an intermediate in biotin synthesis (17, 27, 48). *E. coli* contains two genes, *bioC*, which is located in the *bio* operon, and *bioH*, which is unlinked to the other *bio* genes, that appear to be involved in the early steps of biotin biosynthesis leading up to pimeloyl-CoA, but their exact roles are unknown (14, 32). Although there are no obvious homologs of *bioC* or *bioH* in the two sequenced *bio* operons of *B. sphaericus*, Lemoine et al. (32) have suggested that both the BioC protein of *E. coli* and the BioX protein of *B. sphaericus* may function as acyl carrier proteins involved in pimeloyl-CoA synthesis. Like most acyl carrier proteins, BioX possesses a consensus sequence for a phosphopantetheine attachment site. BioC does not possess such an attachment site; however, Lemoine et al. (32) proposed that BioC functions in a way similar to that of chalcone synthase, an enzyme which does not require the 4′-phosphopantetheine group. They have also identified a consensus sequence in BioH protein which is characteristic of acyltransferase and thioesterase proteins.

Prior to this work, little was known about the biotin biosynthetic genes in *Bacillus subtilis*. Pai (40) had isolated a collection of biotin auxotrophs and shown that they all map at the same locus on the chromosome (262°) and are weakly linked to *aroG* by transformation. On the basis of nutritional requirements and excreted products, the mutants could be divided into three classes that appeared to correspond to *E. coli* mutations in *bioB*, *bioA*, and *bioF* (17, 40). Here we report that the *bio* genes of *B. subtilis* are located in a single operon and that genes with similarity to *bioW*, *bioA*, *bioF*, *bioD*, and *bioB* are found in this operon. In addition, the *B. subtilis* operon contains two other genes that correspond to no other known *bio* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are listed in Table 1. Plasmids pUC9 (57), pUC19 (61), pCL1921 (33), pJGP44, and pTR264 (31) were used for cloning into *E. coli*. pJGP44 is a derivative of pBR322 that contains an 82-bp polylinker with multiple restriction sites inserted between the filled *Eco*RI site and the *Nru*I site of pBR322 (6a). *E. coli* strains were grown on Luria-Bertani medium without glucose. Competent *E. coli* was prepared by the method of Inoue (28) or purchased from Bethesda Research Laboratories, Inc. *E. coli* cells transformed by electroporation were prepared, stored, and transformed as described by Dower et al. (12). *B. subtilis* cells were grown on Tryptose Blood Agar Base (Difco) plates or in veal infusion broth-yeast extract (VY) broth (7). Competent *B. subtilis* was prepared, stored, and transformed as described by Dubnau and Davidoff-Abelson (13). Plasmid

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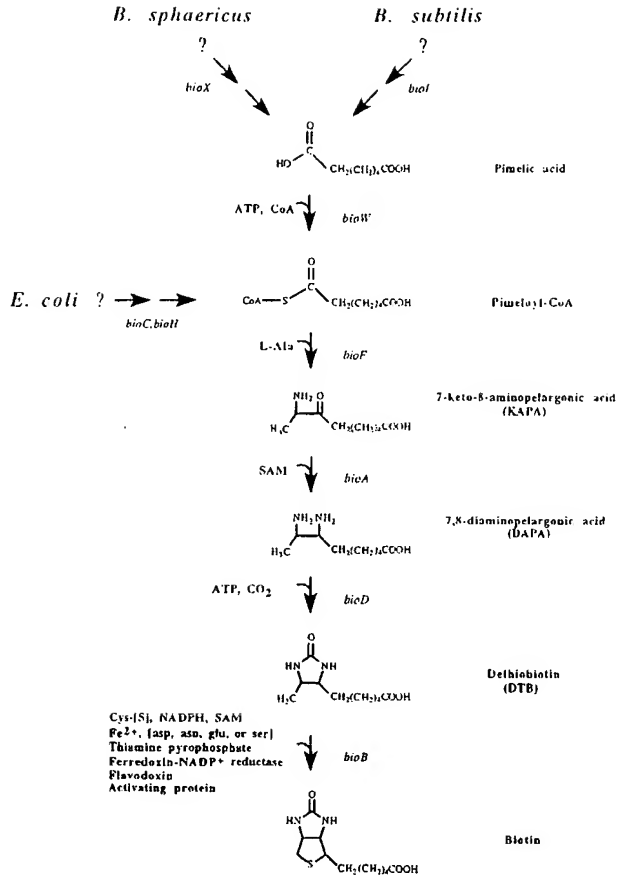

FIG. 1. Biotin biosynthesis pathways in *E. coli*, *B. subtilis*, and *B. sphaericus*. The question marks indicate that the pathways for the synthesis of the intermediates pimeloyl-CoA in *E. coli* and pimelic acid in *B. subtilis* and *B. sphaericus* are not known. The last reaction is catalyzed by the *bioB* gene product; the potential sulfur donor cysteine (Cys-[S]) and the additional proteins and cofactors listed are based on in vitro studies using *E. coli* cell extracts (6, 15, 25, 26). asp, aspartate; asn, asparagine; glu, glutamate; ser, serine; L-Ala, alanine; SAM, *S*-adenosyl-*L*-methionine.

DNA from *E. coli* was prepared by using purification kits purchased from Qiagen, Inc.

Cloning of the biotin operon. The positive selection vector pTR264 (31) was used to construct a library of ~8- to 10-kb fragments of *B. subtilis* GP208 DNA in *E. coli*. Clones with inserts were selected by plating transformants on Luria-Bertani plates with tetracycline (10 μg/ml).

pTR264, prepared in *E. coli dam* mutant strain GM48 and digested with *Bcl*I, was ligated with chromosomal DNA from *B. subtilis* GP208 which had been partially digested with *Sau*3A and fractionated on a sucrose gradient (8- to 12-kb fragments). *E. coli* biotin mutants R879 (*bioA*), R875 (*bioB*), R878 (*bioC*), R877 (*bioD*), and R872 (*bioF*) were each transformed with the ligated DNA by electroporation, and Bio^+ colonies were selected on BIOS medium (7). Bio^+ transformants that were also Tc^r were analyzed for plasmid content.

Cloning of a *B. subtilis* fragment containing the 5′ end of the *bio* operon. Analyses of restriction maps and Southern blot data using a *bioW*-containing fragment from pBIO100 as a probe indicated that a 5.5-kb *Pst*I fragment would contain a complete *bioA* gene and ~2.7 kb of upstream sequences (data not shown). A plasmid, pBIO116, containing this fragment was subsequently recovered when a mini plasmid library of 4.4- to 6.6-kb *Pst*I fragments of *B. subtilis* chromosomal DNA was transformed into *E. coli* BI259 (*bioA pcnB*) and Bio^+ colonies were selected. pBIO116 transformed BI259 again to biotin prototrophy at a high frequency but did not transform R879 (*bioA pcnB*$^+$) to either biotin prototrophy or ampicillin resistance.

Only limited quantities of pBIO116 were recovered from the *pcnB* strain. The *pcnB80* allele which was used in this cloning experiment is reported to reduce the copy number of pBR322 replicons to about 6% of wild-type yields (34). To improve plasmid yields without impairing plasmid stability, the unique *Bam*HI site in the 3′ end of *bioW* was used to subclone a 2.8-kb *Bam*HI-*Pst*I fragment from pBIO116 into a low-copy-number plasmid, pCL1921 (33). A plasmid, pBIO350, that contained the correct 2.8-kb *Bam*HI-*Pst*I fragment was recovered. The quantity of pBIO350 recovered from this strain was significantly higher than that of pBIO116 isolated from the *pcnB80* strain.

Construction of deletions in the biotin operon. The 10-kb *Eco*RI-to-*Bam*HI fragment that contained most of the *bio* operon (except for part of *bioW* and the promoter) was cloned from *B. subtilis* GP275 (an isogenic strain of GP208) into *Eco*RI- and *Bam*HI-digested pJGP44 to give pBIO201. Several deletion mutants and subclones were made from pBIO201 in order to roughly locate the *B. subtilis bio* genes corresponding to the known *E. coli bio* genes by complementation. Deletions were made by cutting with the appropriate restriction enzyme, filling in overhangs with Klenow fragment when necessary, and religating. Subclones were made into pUC9.

The 1.5-kb *Eco*RI-to-*Cla*I fragment of pBIO201 was removed to give pBIO202, the 1.6-kb *Eco*RI-to-*Xho*I deletion gave pBIO203, the 4.5-kb *Eco*RI-to-*Asp* 718 deletion gave pBIO204, the 5.2-kb *Eco*RI-to-*Sma*I deletion gave pBIO205, and the 7-kb deletion from *Eco*RI to the rightmost *Eco*RV gave pBIO206. The 4.3-kb *Bam*HI-to-*Sma*I deletion gave pBIO207, the 3.6-kb insert *Hin*dIII-to-polylinker *Hin*dIII deletion gave pBIO208, and the 3.9-kb *Bgl*II-to-*Bgl*II deletion gave pBIO209. The 2.6-kb central *Pst*I subclone gave pBIO210, the central 4.1-kb *Eco*RV subclone (into the *Sma*I site of pUC9) gave pBIO211, and the 3.3-kb *Eco*RI-to-*Eco*RV subclone (into the *Eco*RI-to-*Sma*I backbone of pUC9) gave pBIO212.

Construction of clones of *bioI* and/or *orf2*. Copies of *bioI* and *orf2* were generated by PCR using a Boehringer Mannheim PCR kit. A *Hin*dIII site was introduced at the 5′ end of each gene, a *Bam*HI site was introduced at the 3′ end of *bioI*, and an *Asp*718I site was introduced at the 3′ end of *orf2*. The PCR-generated fragments were each cloned into three plasmids with different copy numbers, i.e., the low-copy-number plasmid pCL1921; a medium-copy-number plasmid, pJGP44; and the high-copy-number plasmid pUC19. In two of these recombinant plasmids expression of *bioI* and *orf2* is under the control of the *lac* promoter (pCL1921 and pUC19).

DNA sequencing. The *B. subtilis bio* genes contained on clones pBIO100 and pBIO350 were sequenced by the Sanger dideoxy sequencing method using Sequenase kits, version 2.0 (United States Biochemicals, Cleveland, Ohio) as instructed by the manufacturer. The strategy used to obtain the DNA sequence of the 8- to 10-kb region was to divide the region into four plasmid subclones of approximately 2 to 3 kb and then make nested sets of deletions progressing through each subclone. To generate the nested deletions, the exonuclease III-endonuclease S1 method was used; the reagents were purchased as a Generase kit (instructions included; Promega, Madison, Wis.). Nested deletions were made from both ends for three of the subclones and from one end for the fourth. Sequencing both sets of nested deletions for three of the subclones gave the sequence of both strands of each subclone. For pBIO350, one strand was determined similarly and the opposite strand was determined by synthesizing sequencing primers at intervals of approximately 150 bp. The junctions between nonoverlapping subclones were confirmed by sequencing from synthetic primers using pBIO201 or pBIO100 (or subclones thereof) as a template. The sequences were aligned and compared with the DNASTAR computer program (DNASTAR, Inc., Madison, Wis.).

Construction of *cat* insertions. A *cat* cassette, encoding chloramphenicol resistance, derived from pMI1101 (62) was inserted by ligation into the coding region of *bioW* by using a *Bam*HI site; between the *Bsp*EI and *Pml*I sites, deleting 260 bp of *bioB*; into *bioI* by using a *Sma*I site; between a pair of *Sst*I sites, deleting 457 bp of *orf2* plus 149 bp of downstream sequences; into *orf3* by using an *Xmn*I site; into *orf6* by using an *Eco*RV site; and between the pair of *Bst*BI sites, deleting *orf4*. The *cat* cassette was also used to entirely replace the *bio* promoter region by ligating it between the *Hpa*I sites. In each of the *orf2-Sst*I, *orf4-Bst*BI, and *bioB–Bsp*EI-*Pml*I constructions, the *cat* gene was inserted in only one direction. In all other constructions, two different plasmid derivatives, in which the *cat* cassette was inserted in either possible orientation, were generated. Each of these mutations was then integrated into the *bio* locus by first linearizing the *cat*-containing plasmid by a restriction enzyme cut outside of the *bio* DNA; then transforming this cut DNA into a competent prototrophic *B. subtilis* strain, PY79; and then selecting for chloramphenicol resistance (Cm^r) at a final chloramphenicol concentration of 5 μg/ml.

Construction of a *bioW-lacZ* fusion. To construct a *bioW-lacZ* translational fusion, a 3.1-kb *Bam*HI-to-*Bgl*II fragment containing most of the coding region of *E. coli lacZ* (amino acid residues 24 to 1021) was ligated into the *Bam*HI site of pBIO350, to give pBIO397. The *bioW-lacZ* fusion was then used in the construction of a second plasmid to allow integration of the fusion into the modified SPβ prophage SPβc2*del*2::Tn*917*::pSK10Δ6 (63). To bring about this integration, the following four DNA fragments were ligated together to generate plasmid pBIO407: a 6-kb *Pst*I-to-*Kpn*I fragment of pBIO397 containing the *bioW-lacZ* fusion, a PCR-generated 2-kb *Kpn*I-to-*Bam*HI fragment containing the *oriC* and *repA* region of pCL1921, a PCR-generated 1.2-kb *Pst*II-to-*Sal*I fragment containing the *cat* gene of pC194 (22), and a PCR-generated *Sal*I-to-*Bgl*II fragment containing the pUC9 *bla* gene. pBIO407 contains the *bla*, *lacZ*, and selectable *cat* genes in the appropriate orientation to allow integration of the *bioW-lacZ* fusion into the SPβc2*del*2::Tn*917*::pSK10Δ6 prophage of ZB493 (63). A specialized transducing lysate containing SPβ::*bioW-lacZ* was obtained by heat induction at 50°C.

TABLE 1. Bacterial strains used in cloning, complementation, and analysis of *B. subtilis bio* genes

Strain	Relevant genotype or description	Source or reference(s)
B. subtilis		
PY79	SPβ^c prototroph	62
BI421	*birA*	7
JKB3173	*bioA173 aroG932*	17, 40
BGSC1A92	*bioB141 aroG932 sacA321 argA2*	*Bacillus* Genetic Stock Center
JKB3112	*bioF112 aroG932*	17, 40
GP208	*leu amyE* Δ*apr* Δ*npr* Δ*isp-1* (Met$^-$)	49
GP275	*leu amyE* Δ*apr* Δ*npr* Δ*isp-1* (Met$^-$) Δ*epr* Δ*bpr* Δ*mpr* Δ*hpr*	50
ZB493	*urpC2 pheA1 abrB703* SPβc2*del2*::Tn*917*::pSK10Δ6	63
E. coli		
YMC9	Δ*lacU169 endA1 hsdR17 supE44 thi-1*	4
DH5α	F$^-$ (f80d*lacZ*Δ*M15*) Δ*lacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1*	Bethesda Research Laboratories
GM48	F$^-$ *thr leu thi lacY galK galT ara fhuA tsx dam dcm supE44*	New England Biolabs
R872	*bioF3*	8
R875	*bioB17*	8
R877	*bioD19*	8
R878	*bioC18*	8
R879	*bioA24*	8
BM7086	Δ(*mal-bioH*) *gal*	19
BI259	*bioA24 pcnB80*	This study

Partial diploids were generated by transforming the appropriate Bio$^+$ *B. subtilis* strain to Cm^r with the *cat*-containing transducing phage. These partial diploids were then grown in Spizizen's minimal salts medium (52) containing 0.4% glucose and 0.04% sodium glutamate in the presence or absence of biotin (10 μg/liter). Samples were harvested at mid-exponential phase for *o*-nitrophenyl-β-D-galactoside assay (35).

Nucleotide sequence accession number. The DNA sequence of 10.2 kb including the *bio* operon has been submitted to GenBank under accession number U51868.

RESULTS AND DISCUSSION

Cloning of the *B. subtilis* biotin genes. A plasmid library of random *B. subtilis* partial *Sau*3A fragments (~8 to 12 kb) was constructed in *E. coli* by using the positive selection vector pTR264 as described in Materials and Methods. The library was used to transform *E. coli bio* mutants R879 (*bioA24*), R875 (*bioB17*), R878 (*bioC23*), R877 (*bioD19*), R872 (*bioF3*), and BM7086 (Δ*malA-bioH*) (8, 19). Bio$^+$ transformants containing plasmids that complemented each *E. coli bio* mutation were recovered. Plasmids pBIO100 and pBIO101 were isolated by complementation in R879 (*bioA*); plasmids pBIO102 and pBIO103 were isolated by complementation in R877 (*bioD*); plasmid pBIO104 was isolated by complementation in R872 (*bioF*); plasmids pBIO109 and pBIO110 were isolated by complementation in BM7086 (Δ*bioH*); and plasmids pBIO111 and pBIO112 were isolated by complementation in R878 (*bioC*). Initial restriction analysis of the isolated plasmids indicated significant overlap of the cloned DNA fragments, suggesting that the *B. subtilis* biotin locus contains genes functionally equivalent to the *E. coli* genes *bioA*, *bioC*, *bioD*, *bioF*, and *bioH* (Fig. 2). pBIO100 extended the farthest to the right,

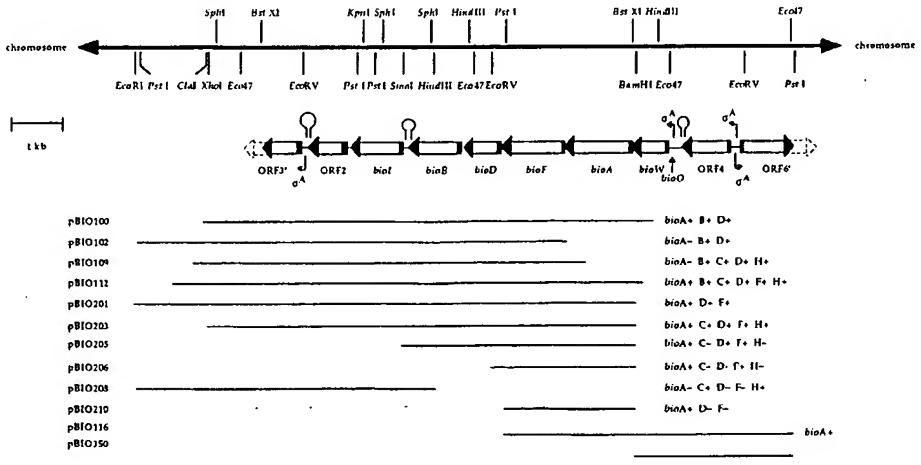

FIG. 2. Physical map of the *B. subtilis bio* operon and flanking DNA. The locations of the structural genes, the putative promoter, the regulatory regions, and the transcription termination sites were determined from the nucleotide sequence of the 10.2-kb *Bst*XI-*Pst*I DNA region. Assignment of the *bio* genes is described in the text. Complementation of *E. coli bio* mutants by plasmids containing cloned fragments of the *B. subtilis bio* operon and flanking regions is indicated by plus signs; no complementation is indicated by minus signs. Mutations not listed were not tested. Endpoints of DNA segments carried by pBIO100, pBIO109, and pBIO112 are approximate. Symbols: ▭, ORF; ▮, *Bacillus* RBS; ♀, putative rho-independent transcription termination site; ⌝, possible start site of transcription for a σ^A-recognized promoter.

~300 bp beyond the unique *Bam*HI site at the right end of the restriction map of the *bio* locus shown in Fig. 2. pBIO110 extended the farthest to the left, ~1,100 bp beyond the *Eco*RI site at the other end of the restriction map (data not shown). Southern blots indicated that the insert DNA of pBIO100 was derived from a single continuous segment of the *B. subtilis* chromosome (data not shown).

Complementation and marker rescue of *B. subtilis* and *E. coli bio* mutants with plasmids containing *B. subtilis bio* genes. To confirm that the cloned DNA of pBIO100 contained *B. subtilis bio* genes, pBIO100 was tested for the ability to marker rescue *B. subtilis bio* mutations (40). The plasmid restored biotin prototrophy to *bioA*, *bioB*, and *bioF* mutants at high frequencies, indicating that the cloned DNA contained all or part of each of these *B. subtilis bio* genes. Several of the pBIO plasmids were also examined for their ability to complement *E. coli* strains with mutations in *bioA*, *bioB*, *bioC*, *bioD*, *bioF*, or *bioH*. Most plasmids complemented more than one *E. coli* biotin mutation (Fig. 2). The isolate pBIO112 complemented *E. coli* mutations in *bioA*, *bioB*, *bioC*, *bioD*, *bioF*, and *bioH* (Fig. 2); however, pBIO112 did not complement the *E. coli* Δ(*gal-uvrB*) mutation, which removes the entire *E. coli bio* locus.

The 9.9-kb *Eco*RI-to-*Bam*HI fragment containing most of the *bio* locus was cloned into a derivative of pBR322, pJGP44, resulting in plasmid pBIO201. To perform complementation experiments with plasmids with defined endpoints, a series of deletions was generated from pBIO201 as described in Materials and Methods. Each deletion-carrying plasmid was introduced into five *E. coli bio* mutants (*bioA*, *bioC*, *bioD*, *bioF*, and *bioH*), and complementation was scored. As shown in Fig. 2, the *B. subtilis bio* genes complementing these *E. coli* genes were located in the 8-kb fragment of DNA from *Bam*HI to *Xho*I. The removal of 5.4 kb from the left of the pBIO201 insert (pBIO205) eliminated the ability to complement *bioC* and *bioH* mutants. pBIO206 contained only the rightmost 2.5 kb of the biotin cluster and complemented only *bioA* and *bioF* mutants. One clone, pBIO208, in which the rightmost 4.0 kb of insert DNA was deleted complemented *E. coli bioC* and *bioH* mutants but failed to complement *E. coli bioA*, *bioD*, or *bioF* mutants. These results suggested the gene order (*bioC*, *bioH*)-*bioD*-*bioF*-*bioA*.

Cloning of a *B. subtilis* fragment containing the 5′ end of the *bio* operon. As described below, DNA sequences of the rightmost end of the cloned insert (pBIO100) that extended furthest to the right revealed about 300 bp of an open reading frame (ORF) that was homologous to *B. sphaericus bioW*, the gene encoding pimeloyl-CoA synthase (17, 43), followed immediately by genes with strong similarity to *bioA*, *bioF*, *bioD*, and *bioB* from *E. coli* and *B. sphaericus* (Fig. 2). The 5′ end of *bioW* and the promoter of the *bio* operon were not present on any of the originally cloned DNA fragments. Suspecting that it might be difficult to clone this region in high-copy-number plasmids, we cloned DNA fragments containing *bioA* and the adjacent upstream region by complementation in an *E. coli* strain containing a *bioA* mutation and a *pcnB* mutation to reduce plasmid copy number (34) as described in Materials and Methods.

Identification and organization of *bio*-specific coding regions and transcriptional regulatory signals. Analysis of ~10 kb of the DNA sequence from pBIO100 and pBIO350 indicated that many or all of the *B. subtilis* biotin biosynthetic genes are located in a single operon containing seven coding regions (Fig. 2). The *bioW* gene appears to be the first gene in the operon. Approximately 84 bp upstream from *bioW* is a putative vegetative (σ^A) promoter sequence (TTGACA—17 bp—TATATT [36]). This probable promoter sequence was followed by a 33-bp segment with strong sequence homology to the "regulatory" sites of the *B. sphaericus bio* operons and lesser similarity to the *E. coli bio* operator site. Comparison of the nucleotide sequences of this region with those of the 5′ noncoding region of the *B. sphaericus bioDAYB* operon (51) revealed two clusters of conserved nucleotides (13 and 11 bp) separated by a nonconserved 9-bp segment (Fig. 3).

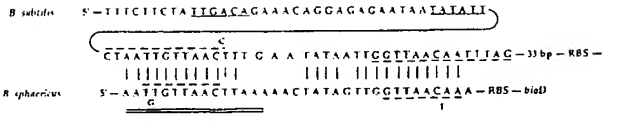

FIG. 3. Comparison of the nucleotide sequences of the *B. sphaericus bioDAYB* regulatory region and the putative *B. subtilis bio* promoter and regulatory region. The upper sequence represents the putative *B. subtilis bio* promoter and regulatory region. The lower sequence represents the *B. sphaericus bioDAYB* regulatory region (17). The sequence shown spans nucleotides 1995 to 2072 of the nucleotide sequence submitted to GenBank (accession no. U51868 [see Materials and Methods]). Symbols: double bold underline, 15-bp putative regulatory region of *B. sphaericus bioDAYB*; dashed lines, regions of dyad symmetry; single bold underlines, −35 and −10 regions of a possible promoter. RBS, putative *Bacillus* RBS. The nucleotides above or below the sequences were displaced to facilitate sequence alignment.

The *bioW* gene (259 amino acids) is followed by ORFs with homology to *bioA* (448 amino acids), *bioF* (389 amino acids), *bioD* (231 amino acids), and *bioB* (335 amino acids) (Table 2). The next two ORFs, *bioI* (395 amino acids) and *orf2* (253 amino acids), showed no sequence similarity to *bioC* or *bioH* or to any other known *bio* gene (Fig. 2 and Table 2). Comparison with the protein database of GenBank, however, indicated significant similarity of the deduced amino acid sequence of *bioI* to those of cytochrome P-450 enzymes from *Bacillus megaterium* ($P\text{-}450_{BM\text{-}1}$ [21]), *Saccharopolyspora erythraea* (EryF [20] and EryK [53]), and other organisms (53). Cytochrome P-450s include monooxygenases known to catalyze hydroxylation of many different kinds of substrates, including fatty acids. Since synthesis of pimelic acid, a precursor to biotin, might involve hydroxylation and/or further oxidation of a fatty acid, *bioI* may be involved in an early step in biotin synthesis (see below). Although similar protein database searches did not reveal a specific function for the *orf2* gene product, significant similarity between the N-terminal end of the deduced protein and putative NAD or NADH binding sites of short-chain alcohol dehydrogenases (e.g., BphB [3, 55]), dehydratases (e.g., RfbB [30]), and the β-ketoreductase domain of $EryA_{II}$ of *S. erythraea* (11) was detected. Since this region of Orf2 also contains a GXGXXG motif, which is characteristic of a FAD or NAD binding site (60), it is conceivable that *orf2* encodes an NADH- or NADPH-dependent enzyme.

Each gene in the *bio* operon is preceded by a ribosome binding site (RBS), with calculated ΔGs ranging from −10.8 to −18.6 kcal (ca. −45.2 to −77.8 kJ)/mol (Table 2). All genes are oriented in the same transcriptional direction (right to left). In addition, the 5′ ends of *bioA*, *bioF*, *bioD*, and *bioB* overlapped the 3′ ends of the genes preceding them, suggesting that expression of these genes could be regulated, in part, by translational coupling. *bioI* and *orf2* are separated from the genes that precede them by 68- and 67-bp intercistronic regions, respectively.

orf2 appears to be the last gene in the *bio* operon, as it is immediately followed by a region of dyad symmetry resembling a rho-independent transcription termination site (ΔG = −15.4 kcal [ca. −64.4 kJ]/mol). Another stem-loop structure with terminator-like features was detected in the region between

TABLE 2. Enzymes, genes, and regulatory elements of the *B. subtilis bio* operon and flanking DNA.

Gene	RBS ΔG (kcal/mol)[a]	Predicted start codon	Enzyme or function	Calculated no. of amino acids	Estimated M_r	% Amino acid identity to corresponding gene product from:		
						E. coli[b]	*B. sphaericus*[c]	Other
bioW	−10.8	ATG	Pimeloyl-CoA synthase	259	29,633		44	
bioA	−15.8	ATG	DAPA aminotransferase	448	50,118	34	44	
bioF	−11.6	TTG	KAPA synthase	389	42,567	35	50	
bioD	−18.6	TTG	DTB synthetase	231	25,114	29	28	
bioB	−12.2	ATG	Biotin synthase	335	36,931	34	71	22[d]
bioI	−18.4	GTG	Cytochrome P-450	395	44,838			30,[e] 33[f]
orf2	−17.6	GTG	Unknown	253	28,204			
orf3	−20.0	GTG	Unknown membrane-associated transport protein	>258	>28,600	53,[g] 24,[h] 23[i]		
orf4	−10.0	ATG	Unknown	299	33,780			
orf6	−17.4	ATG	Unknown regulatory protein	>266	>29,200	30,[j] 26[k]		

[a] Calculated according to the method of Tinoco et al. (56). One kilocalorie equals 4.184 kJ.
[b] Identity to *E. coli bio* gene products (38).
[c] Identity to *B. sphaericus bio* gene products (17).
[d] Identity to *E. coli lipA* product (44).
[e] Identity to *B. megaterium* cytochrome P-450$_{BM-1}$ (21).
[f] Identity to *S. erythraea eryF* product (20).
[g] Identity to *B. subtilis lplC* product (18).
[h] Identity to *E. coli malG* product (10).
[i] Identity to *E. coli ugpE* product (39).
[j] Identity to *E. coli ebgR* product (54).
[k] Identity to *E. coli purR* product (45).

bioB and *bioI*. Several secondary structures of the mRNA are possible, with the most favored structure having a ΔG of formation of −11 kcal (ca. −46 kJ)/mol and the least favored structure having a ΔG of −5.6 kcal (ca. −23 kJ)/mol. Northern (RNA) blots indicated that both terminator-like regions are functional: two steady-state transcripts originating near the putative P_{bio} promoter were detected, i.e., a 7-kb RNA that corresponds to the predicted transcript for the entire seven-gene operon and a 5-kb transcript that corresponds to the first five genes in the operon (41). The steady-state levels of the 5-kb transcript were, however, about eightfold greater than the levels of the full-length transcript, suggesting that the terminator-like structure between *bioB* and *bioI* serves to limit expression of *bioI* (41).

Downstream from the end of the biotin operon, a strong RBS (ΔG = −20.0 kcal [ca. −84 kJ]/mol) and 260 amino acids of another coding region, *orf3*, were found. The remainder of *orf3* continues beyond the *Bst*XI site which marks the end of the sequenced region. *orf3* is preceded by a sequence, TGAT AACGCTTACA, with a perfect match to the consensus sequence TG(T/A)NANCGNTN(A/T)CA for catabolite-controlled genes in *B. subtilis* (24, 58). The deduced amino acid sequence of *orf3* showed significant similarity to a number of *E. coli* membrane-associated transport proteins, e.g., glycerol-3-phosphate permease (UgpE [39]) and maltose permease (MalG [10]). In particular, the partial Orf3 protein contains a 20-amino-acid sequence common to all membrane-associated transport proteins (10). Significant homology (>50%) of Orf3 protein to LplC, a transmembrane protein of *B. subtilis*, was also found (18).

Upstream from the biotin operon is a coding region, *orf4*, preceded by an RBS and a putative σ^A promoter (Table 2). *orf4* is followed by a region of dyad symmetry that resembles a rho-independent transcription termination site; this possible terminator is approximately 160 bp upstream from the proposed *bioW* start codon. Finally, further upstream from *orf4*, oriented in the opposite direction, is an ORF, *orf6*, extending 266 codons to the limit of the DNA sequencing. *orf6* is preceded by an RBS and a potential σ^A promoter. The deduced amino acid sequence of *orf6* showed significant similarity to those of a number of regulatory proteins of the *E. coli* LacI family, e.g., *E. coli* EbgR (54) and PurF (a repressor of the purine nucleotide biosynthetic operon) (45).

The gene-enzyme relationship, the enzyme size, and the percent(s) homology to the same enzyme from other organisms for each *bio* gene or *orf* are summarized in Table 2.

Construction and analysis of a *bio-lacZ* translational fusion. A translational *lacZ* fusion to *bioW* was constructed to assess the activity and regulation of the putative promoter and regulatory region. This was accomplished by replacing the 3′ end of the *bioW* coding sequence with a 3.1-kb *Bam*HI-*Bgl*II fragment containing a promoterless *lacZ* coding region in a plasmid designed to allow integration into a modified SPβ prophage (see Materials and Methods). This plasmid, pBIO407, contains DNA extending to a position located about 2 kb upstream of the presumed *bioW* start codon and most of the *bioW* coding sequence fused to *lacZ* on a low-copy-number plasmid. pBIO407 turns *lacZ E. coli* colonies pale blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates, suggesting that the fusion is expressed at a relatively low level in *E. coli*.

To test the expression of the *bioW-lacZ* fusion, the fusion was introduced as a single copy into a *B. subtilis* protrotroph (PY79) and a similar strain (BI421) containing a mutation in the unlinked *B. subtilis birA* gene (7), a gene with similarity to the *E. coli birA* gene whose product serves as both the repressor for the biotin operon and the ligase that biotinylates acetyl-CoA carboxylase (5, 9). SPβ specialized transducing phage (63) carrying *bioW-lacZ* was constructed and used to insert the fusion into the chromosome of PY79 and BI421 as described in Materials and Methods. The resulting Bio$^+$ partial diploids were grown in the presence or absence of biotin. As judged by the levels of β-galactosidase activity, the levels of SPβ::*bioW-lacZ* expression were very low, but this expression showed biotin-specific regulation (Table 3). β-Galactosidase activity was repressed by about 10-fold in the presence of exogenous biotin. In a *birA* mutant strain, constitutive expression of the fusion was observed. However, the level of β-galactosidase

TABLE 3. Biotin-regulated expression of SPβ::*bioW-lacZ* translational fusion

Relevant genotype	β-Galactosidase sp act (Miller units)[a]	
	With biotin[b]	Without biotin
bioW-lacZ bio$^+$	0.05 ± 0.01	0.5 ± 0.06
bioW-lacZ bio$^+$ *birA*	0.8 ± 0.15	0.9 ± 0.07

[a] Data are averages ± standard deviations for two isolates and two assays each (calculated according to the method of Miller [35]).
[b] Biotin was present at 100 μg/liter.

activity in the *birA* strain was only somewhat higher than the levels observed in PY79 containing SPβ::*bioW-lacZ* and grown under nonrepressing conditions. Similar results were obtained when a *bioW-lacZ* fusion was introduced by integration of a circular plasmid (pBIO397*cat*) by Campbell-like recombination at the *bio* locus (data not shown). These results suggest that the *B. subtilis bio* promoter is regulated by *birA* and biotin, as is the case for the divergent *bio* promoters of *E. coli*. In future work, it will be interesting to establish whether the *B. subtilis bio* operon is regulated by the *B. subtilis* BirA by a repressor-operator mechanism similar to that used in *E. coli* for the regulation of biotin biosynthesis.

The *B. subtilis bioI* gene complements both *E. coli bioC* and *bioH* mutants. The presence of two genes, *bioI* and *orf2*, with homology to neither *bioC* nor *bioH* of *E. coli*, raised the issue of which gene(s) was complementing which *E. coli* mutant. Complementation studies using plasmid subclones that contained either *bioI* or *orf2* alone under the transcriptional control of the *lacZ* promoter (see Materials and Methods) indicated that *bioI* alone was sufficient to complement both *E. coli* BM7086 (Δ*bioH*) and *E. coli* R878 (*bioC*). Plasmids containing *orf2* did not give normal complementation of either *E. coli* BM7086 or *E. coli* R878. The cytochrome P-450-like product of the *bioI* gene of *B. subtilis* can apparently supply an activity needed for biotin synthesis that can substitute for, or bypass, the activity missing in either *bioC* or *bioH* mutants of *E. coli*.

Insertional mutagenesis of the *bio* operon and flanking coding regions. To verify the boundaries of the *bio* operon predicted from the nucleotide sequence and to confirm the roles of previously unidentified *bio* genes, a *cat* cassette (chloramphenicol resistance gene) was used to construct insertions or deletions in *bioW*, *bioB*, *bioI*, *orf2*, the *bio* promoter region, *orf3*, *orf4*, and *orf6*. First, plasmid derivatives containing these mutations were constructed in *E. coli*, and then the *cat* insertions were transferred to the *bio* locus of *B. subtilis* by DNA transformation (see Materials and Methods). The locations of these mutations are diagrammed in Fig. 4. As summarized in Table 4, insertions into *orf3* and *orf6* and deletion of *orf4*, which represent mutation of the coding regions located outside of the predicted *bio* operon, generated Cm^r, prototrophic colonies. Insertions and deletions in the *bio* operon gave results that generally supported the conclusions from the nucleotide sequence data. Replacement of the region upstream of *bioW* containing the putative P_{bio} promoter with the *cat* gene oriented opposite to the biotin operon and interruption of *bioW* with the *cat* gene oriented in either direction relative to the *bio* operon generated an unambiguous Bio$^-$ phenotype. However, replacement of the putative P_{bio} promoter region with the *cat* gene inserted in the same transcriptional direction as the biotin operon generated Bio$^-$ cells that reverted to Bio$^+$ at a high frequency (0.1%). Bioassay experiments indicated that biotin vitamer production from such a Bio$^+$ revertant was increased in the presence of low concentrations of chloramphenicol, suggesting that expression of the biotin operon resulted from read-through transcription from the chloramphenicol-inducible *cat* promoter. We also observed that the *bioB* gene was expressed when the *cat* gene was inserted into the biotin operon upstream of *bioB* and oriented in the same transcriptional direction, as judged by growth of such bacteria on DTB (Table 4).

Deletion (*Pml*I to *Bsp*BI) of the 3′ end of *bioB* also generated a Bio$^-$ phenotype, confirming that *bioB* was required for biotin biosynthesis. However, the 3′ end of the operon could not be definitively identified by this genetic method. Insertions into *bioI* resulted in Cm^r colonies that were partially deficient in biotin production, i.e., that grew poorly on biotin-free medium but grew as well as wild-type colonies in the presence of biotin (33 μg/ml), whereas the *orf2*::*cat* mutation gave Bio$^+$ colonies. These results suggested that *bioI* is not absolutely required for biotin production and that the *orf2* gene product is dispensable for biotin biosynthesis. The question of whether *orf2* encodes a redundant enzyme that functions in biotin synthesis or simply an unrelated protein awaits further experimentation. The *bioA* gene of *E. coli* is also located in an operon with another ORF (*orf1*) also of unknown function. However,

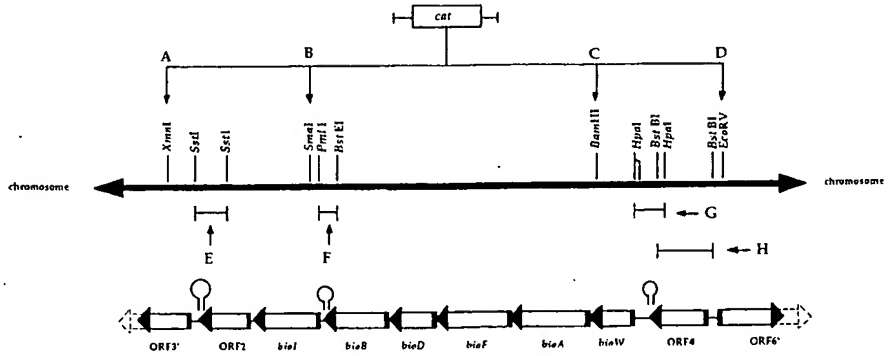

FIG. 4. Locations of *cat*-containing insertions and deletions within the *B. subtilis bio* operon and flanking DNA. As described in Materials and Methods, in vivo mutations of the *bio* genes and flanking open reading frames were generated either by inserting a 1.5-kb *cat*-containing cassette into the indicated restriction site (A, *Xmn*I; B, *Sma*I; C, *Bam*HI; or D, *Eco*RV) or by replacing the indicated region with the *cat* cassette (E, replacement of a 606-bp *Sst*I fragment; F, replacement of a 260-bp *Pml*I-*Bsp*EI fragment; G, replacement of three adjoining *Hpa*I fragments totalling 313 bp; H, replacement of a 966-bp *Bst*BI fragment). Not all restriction sites are shown. *B. subtilis* strains containing these mutations were examined for their biotin phenotypes, and the results are tabulated in Table 4.

TABLE 4. Characterization of insertion and deletion derivatives of the biotin operon

Biotin operon derivative (mutation) and *cat* gene orientation[a]	Biotin phenotype[b]	Growth on[c]:		
		Minimal medium[d]	DTB[e]	Pimelic acid[f]
Wild-type *bio* operon	+	+	+	+
A (Ω*orf3*)				
R	+	+		
L	+	+		
B (Ω*bioI*)				
R	+/−	+/−	+	+
L	+/−			+
C (Ω*bioW*)				
R	−	−	−	−
L	−		+/−	
D (Ω*orf6*)				
R	+	+		
L	+	+		
E (Δ*orf2*)				
R	+	+		
F (Δ*bioB*)				
R	−		−	
G (ΔP_{bio})				
R	−		−	−
L	+[g]		+	+[g]
H (Δ*orf4*)				
L	+	+		

[a] See Fig. 4 for a map of *cat* insertions within the biotin operon. Insertion derivatives having the *cat* gene in either orientation were obtained: R (right) and L (left) identify the transcriptional orientation of the inserted *cat* gene when the *bio* operon is oriented as shown in Fig. 4.

[b] Biotin phenotype determined by patching bacteria on biotin-free agar plates. +, biotin prototroph; +/−, biotin bradytroph; −, biotin auxotroph.

[c] +, prototrophic; +/−, bradytrophic; −, auxotrophic.

[d] Growth of bacteria on Spizizen's minimal medium agar plates.

[e] Growth of bacteria on biotin-free agar plates containing 33 μg of DTB per liter.

[f] Growth of bacteria on biotin-free agar plates containing 33 μg of pimelic acid per ml.

[g] Appearance at a frequency of 0.1% of Bio$^+$ bacteria in which biotin synthesis is inducible by chloramphenicol.

there is no sequence similarity between the *B. subtilis orf2* gene product and the *E. coli orf1* gene product.

The biotin bradytroph phenotype generated by the *bioI*::*cat* mutation appeared to be caused by inactivation of *bioI* rather than by a polar effect because strains with mutations disrupting the downstream gene *orf2* or *orf3* were Bio$^+$. To determine whether the *bioI* gene product was involved in formation of pimelic acid, we examined whether the *bioI*::*cat* mutation could be bypassed by feeding pimelic acid. Derivatives of PY79 containing *bioI*::*cat* with either orientation of the *cat* gene grew as well as wild-type strains on biotin-free medium containing pimelic acid (Table 4). These results confirmed that the *bioI* gene product is involved early in the biotin pathway.

***E. coli* cells expressing the *bioW* gene of *B. subtilis* can utilize pimelic acid to synthesize biotin.** On the basis of homology with the *B. sphaericus bioW* gene, we hypothesized that the *B. subtilis bioW* gene encodes a pimeloyl-CoA synthase (43). To further examine this gene-enzyme relationship, we tested whether *B. subtilis bioW* expression in *E. coli* could be utilized to synthesize biotin from pimelic acid as reported for the *B. sphaericus bioW* (17). First, a fragment containing the *B. subtilis bioW* gene and its promoter was cloned into plasmid pCL1921, generating pBIO403. Next, pBIO403 was introduced into *E. coli* Δ*bioH* or *bioC* mutants and the resulting strains were tested for complementation. *E. coli* does not have a *bioW* homolog, and *bioC* or *bioH* mutants of *E. coli* cannot be rescued for growth on biotin-free medium by the addition of pimelic acid. However, both Δ*bioH* and *bioC* mutants of *E. coli* containing pBIO403 grew in the absence of biotin when, and only when, pimelic acid (30 μg/ml) was added to the medium. This result suggests that *bioW* encodes a pimeloyl-CoA synthase that, in the presence of pimelic acid, can bypass *bioH* and *bioC* in *E. coli*.

Early steps in biotin biosynthesis. The early steps in biotin biosynthesis appear to be different in the gram-negative bacteria, such as *E. coli* and *Serratia marcescens*, and the gram-positive bacteria, such as *B. subtilis* and *B. sphaericus*, two distantly related *Bacillus* species. *E. coli* cannot use free pimelic acid as a precursor for biotin synthesis (14), and ^{13}C labeling experiments indicate that free pimelic acid is not an intermediate in biotin biosynthesis (48). On the other hand, *B. subtilis* and *B. sphaericus* readily use pimelic acid, which is converted to pimeloyl-CoA by pimeloyl-CoA synthase, the product of the *bioW* gene. When supplied with the *bioW* gene from *B. subtilis* or *B. sphaericus* (17, 43), *E. coli* can use pimelic acid to bypass the biotin auxotrophy of *bioC* or *bioH* mutants.

Is pimeloyl-CoA synthase an obligatory part of the biotin biosynthetic pathway in *B. subtilis*, or is it part of an alternative pimelic acid salvage pathway? While the answer to this question is not clear, preliminary experiments indicate that the *bioW* gene product is required for biotin synthesis in *B. subtilis*. Insertion of the *cat* gene in place of the promoter region of the biotin operon, oriented in the same direction as the *bio* operon, yielded Bio$^-$ colonies that reverted to Bio$^+$ at a frequency of 0.1%. Insertion of the same *cat* gene in *bioW*, also oriented in the same direction as the *bio* operon, yielded a nonreverting Bio$^-$ phenotype. However, such mutants were able to grow weakly on DTB or DAPA, indicating that the downstream *bioB* and *bioD* genes were being expressed. Furthermore, cells of *B. subtilis* containing an in-frame deletion within the chromosomal *bioW* gene were also Bio$^-$ but were able to grow well on DTB or DAPA (unpublished results). We cannot rule out the possibility that both of these *bioW* mutations exert a polar effect on *bioF* or *bioA* that is more deleterious than the effect on *bioD* or *bioB*. However, it appears most likely that the pimeloyl-CoA synthase is required for biotin synthesis in *B. subtilis* and that pimelic acid is a bona fide intermediate in biotin synthesis in *B. subtilis*.

On the basis of the cytochrome P-450-like structure of the BioI protein, we hypothesize that *B. subtilis* synthesizes pimelic acid by a pathway different from that of *E. coli*. Since other cytochrome P-450s are capable of oxidizing unsaturated fatty acid (59), we suggest that BioI may function to oxidize the double bond of an unsaturated fatty acid. Since BioI will complement an *E. coli bioC* or *bioH* mutant in the absence of pimeloyl-CoA synthase, we further speculate that the BioI protein can use either a free fatty acid or the CoA thioester of a fatty acid as a substrate to produce pimelic acid or pimeloyl-CoA, respectively.

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Note

Genetic Analysis of an Incomplete *bio* Operon in a Biotin Auxotrophic Strain of *Bacillus subtilis* Natto OK2

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We describe the genetic analysis of the *bio* operon of the biotin auxotrophic *Bacillus subtilis* natto OK2 strain. The OK2 strain would only cross-feed with the *Escherichia coli bioB* mutant and also grew well in medium containing dethiobiotin. Sequencing analysis revealed two significant genetic alterations in the *bioW* and *bioF* genes within the *bio* operon of the OK2 strain. Complementation analysis with *B. subtilis* 168 *bio* mutants demonstrated that only the *bioB* gene could complement, but other *bio* operon genes could not. A *bio*$^+$ transformant, isolated from an OK2 strain, has biotin autotrophy.

Key words: *Bacillus subtilis* natto; biotin operon; *bioB*

The biotin biosynthetic operon in *Escherichia coli* and *Bacillus subtilis* has been well documented at the biochemical and molecular biological levels.[1-4] Analysis reveals that analogous enzymes from the two species are similar, and both operons contain the *bioF* gene encoding 8-amino-7-ketopelargonic acid synthase, the *bioA* gene encoding diaminoperalgonic acid aminotransferase, the *bioD* gene encoding dethiobiotin synthetase, and the *bioB* gene encoding biotin synthetase. However, the early steps of the pathway, namely those involved in the synthesis of pimeloyl-CoA, are quite different. *E. coli* contains two genes; *bioC*, which is located in the *bio* operon, and *bioH*, which is not linked to the other *bio* genes, but the roles of these two genes have yet to be identified. On the other hand, *B. subtilis* contains the *bioW* gene encoding pimeloyl-CoA synthetase, which is also found in *Bacillus sphaericus*,[5] and the *bioI* gene, which shows no homology to either *bioC* or *bioH* but is able to complement in either *bioC* or *bioH* of *E. coli* mutants.[3]

B. subtilis natto is a commercially important microorganism used in the fermentation of soybeans to make "natto", a popular food in Japan. Although DNA-DNA hybridization reveals that the genomic DNA of *B. subtilis* natto strains is highly homologous to that of *B. subtilis* 168[6] the *B. subtilis* natto strain requires biotin for growth. Here we describe the genetic analysis of the *bio* operon in *B. subtilis* natto OK2,[7] a highly transformable strain, and compare it to *B. subtilis* 168. The goal of the study was to construct the hyper biotin producer of *B. subtilis* natto and use it to make "biotin-rich natto".

In order to investigate the biotin biosynthetic pathway in the OK2 strain, cross-feeding experiments were done as described[8] with *E. coli bio* mutants (CGSC, Yale University), using *B. subtilis* 168 as a control. Although *B. subtilis* 168 cross-fed the *E. coli* mutants R872 (*bioF103*), R879 (*bioA24*), R877 (*bioD19*), and R875 (*bioB17*), an OK2 strain cross-fed only by the R875 (*bioB17*) strain (Table 1). Moreover, both strains could not cross-feed an *E. coli* R878 (*bioC23*) strain. These results suggested that an OK2 strain could only convert dethiobiotin into biotin during the last step of the biotin biosynthetic pathway.

To analyze the biotin biosynthetic pathway of the OK2 strain at the molecular level, the *bio* operon derived from chromosomal DNA of the strain was cloned by PCR amplification with primers designed based on the

Table 1. Cross-feeding Tests between *E. coli bio* Mutants and *Bacillus* Strains

E. coli mutants		*Bacillus* strains	
Strain	Genotype	*B. subtilis* 168	*B. subtilis* natto OK2
R878	*bioC23*	−	−
R872	*bioF103*	+	−
R879	*bioA24*	+	−
R877	*bioD19*	+	−
R875	*bioB17*	+	+

Five *E. coli bio* mutants were separately streaked onto biotin-free medium in agar plates in which washed cells of *Bacillus* strains were suspended at a concentration of 6.0×10^5 cells/ml. Cross-feeding under these conditions resulted from diffusion of biotin precursors excreted by the cells in the agar. Cross-feeding interactions were scored after 72 hours. +, growth; −, no growth.

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nucleotide sequences of the *B. subtilis* 168 *bio* operon.[3] The *bio* operon was partially amplified using the following pairs of primers: BW-1 (CATCGGCATGTC-TATGGGAGG) and BA-2 (TAACCGCTCGTTTAAC-CAGG), for *bioW* and *bioA* genes; BF-1 (AACAAGC-GATCCACGAGGTT) and BD-1 (CTCTTCGTCAGT-CACTTCTG), for *bioF* and *bioD* genes; BB-1 (GAAT-CAAGTGGGGGTATGAG) and BI-2 (TTCGGCGG-GGCTGACACTTT), for *bioB* and *bioI* genes, respectively. Nucleotide sequence analysis shows the *bio* operon to have a similar structure to that of *B. subtilis* 168 with both operons arranged on a single operon in the order *bioWAFDB* and followed by two genes, *bioI* and *ytbQ*, (accession number of this sequence in the DDBJ, EMBL, and GenBank nucleotide sequence databases, AB088066) (Fig. 1). The amino acid sequence homologies of these gene products with those of *B. subtilis* 168 are extremely high, as follow: BioW, 98.2%; BioA, 98.2%; BioF, 92.0%; BioD, 97.4%; BioB, 98.8%; BioI, 97.2%. However, we found two significant differences between the *bio* operon of OK2 and that of *B. subtilis* 168. First, a single-base change resulted in the replacement of Cys (TGC) (strain 168) by a stop codon (TGA) (strain OK2) at position 226 in the carboxy-terminus of BioW. Secondly, a 54 bp fragment encoding 18 amino acids in the *bioF* gene of *B. subtilis* 168 was largely deleted in positions from 848 to 901 in the OK2 strain. These results suggested that the *bio* operon in the OK2 strain was genetically defective and therefore showed biotin auxotrophy.

To analyze individual *bio* genes of the OK2 strain, each *bio* gene was tested for its ability to complement *B. subtilis* 168 *bio* mutants. Five *B. subtilis* 168 *bio* mutants (*bioW*, *bioA*, *bioF*, *bioD* and *bioB*) were constructed by insertional mutagenesis according to the method described previously.[3] Five *bio* genes of an OK2 strain were amplified with pairs of primers (Table 2). The amplified DNA product was subsequently digested with appropriate restriction enzymes (Table 2) and cloned into same restriction sites of the expression plasmid pWH1520 (MoBiTec). Each of the composite plasmids was used to transform each of above *bio* mutants of *B. subtilis* 168, respectively and selected for biotin auxotrophy or prototrophy (data not shown). Only plasmids carrying the *bioB* gene from the OK2 strain complemented the *bioB* mutant of *B. subtilis* 168. The other plasmids that carried the *bioW*, *bioA*, *bioF*, and *bioD* genes did not complement and these results were identical to those of the cross-feeding tests described above. Two genes of *bioA* and *bioD* from OK2 were highly homologous to those of *B. subtilis* 168, and seven (K39D, D67N, A201E, E205K, M219I, and S430T) and four (D28E, N29H, H32D, and R145H) amino acids substitutions were detected in the *bioA* and *bioD* genes, respectively. These substitutions seem to be essential for enzyme activity and further studies are now in progress.

In addition, we tested whether the OK2 strain could be used to synthesize biotin from its precursor dethiobiotin. The OK2 strain grew well on biotin-free medium containing dethiobiotin as well as medium containing biotin (Fig. 2). Moreover, when insertional mutagenesis was done on the *bioB* gene of OK2,[3] this mutant did not

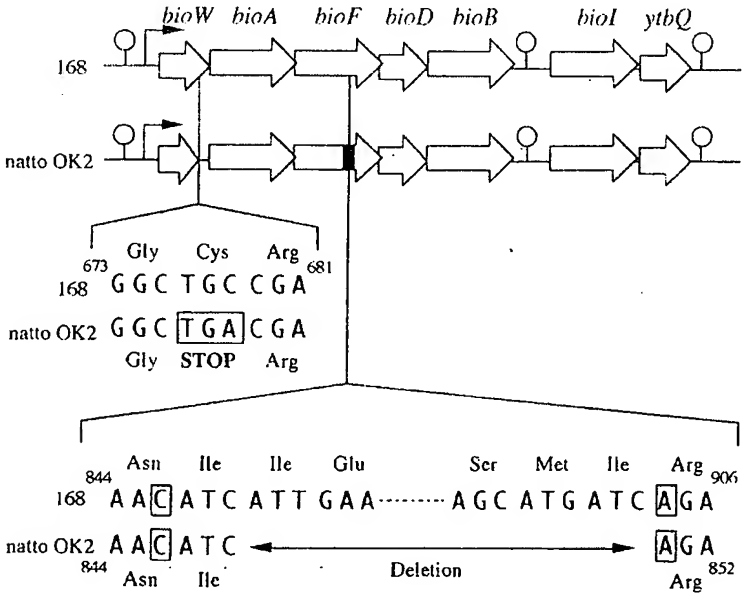

Fig. 1. Structure of the *bio* Operon.

The locations of *bio* structural genes, putative promoter and regulatory regions, and transcription termination sites are shown in the upper diagram. Top set of arrows indicates the *bio* operon of *B. subtilis* 168 and lower set of arrows indicates *B. subtilis* natto OK2. Numbers shown next to the nucleotide sequences indicate distance (bp) from initiation codon. Black box in *bioF* indicates a deletion region. Symbols: arrowhead, open reading frames; ♀, putative transcription termination site; ⌐, possible σ^A-recognized P_{bio} promoter.

Table 2. Nucleotide Sequence of Synthetic Primer Used for PCR

bio gene	Sequence of primer (5′ → 3′; forward and reverse)	Restriction site[a]
bioW	TAGGTACCTAACAATTTAGGTGAGAAG	*Kpn*I
	−57 −39	
	TTAGATCTGGTAAATGGCAGCCAGAGG	*Bgl*II
	714 723	
bioA	ATGGATCCTAAGATGTAAACACGTACATAC	*Kpn*I
	−75 −46	
	CTGCATGCATTGACCGCAGGTTACGATG	*Sph*I
	1294 1313	
bioF	AGGGATCCTGAAGAGCTCTCGGAAATG	*Kpn*I
	−59 −41	
	GAGCATGCGATATAACCGTTTTCCCTAC	*Sph*I
	1103 1132	
bioD	CGGTTAACCATAGTATGGGTGATATTG	*Hpa*I
	−65 −49	
	GAGTCGACCTCATACCCCCACTTGATTC	*Sal*I
	678 698	
bioB	ATACTAGTTGATGAATCAAGTGGGGG	*Spe*I
	−25 −8	
	TAGGATCCCTTTCAGCTTTTCGCAC	*Bam*HI
	995 1012	

[a]The restriction site for cloning has been underlined in the sequence.
Numbers shown on the primer sequence (bold type) are indicated in terms of the distance (bp) from the initiation codon.

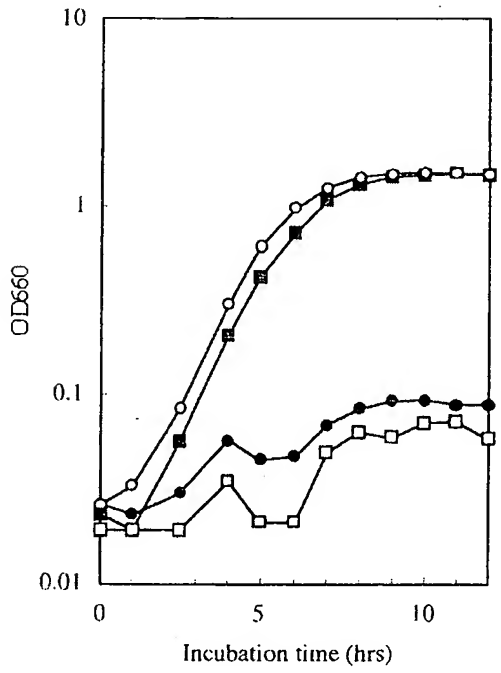

Fig. 2. Growth of *bioB*$^+$ and *bioB*$^-$ Strains of *B. subtilis* Natto OK2.
B. subtilis natto OK2 was grown aerobically at 37°C in minimal medium (14 g of K_2HPO_4, 6 g of KH_2PO_4, 1.9 g of sodium citrate, 2 g of $(NH_4)_2SO_2$, 1.4 g of $MgSO_4 \cdot 7H_2O$, 5 g of glucose and 1 liter of deionized water) with or without biotin (0.1 ng/ml) and dethiobiotin (0.1 ng/ml), respectively. Cell growth was monitored by measuring the optical density at 660 nm. Symbols; ○, *bioB*$^+$ strain with biotin; ■, *bioB*$^+$ strain with dethiobiotin; ●, *bioB*$^-$ strain with dethiobiotin; □, *bioB*$^+$ strain without biotin.

grow on medium containing dethiobiotin (Fig. 2). These results confirm that the *bioB* gene product in the OK2 strain is indeed involved in the last step of the biotin biosynthetic pathway.

The results of this study using *B. subtilis* are similar to those of Hatakeyama *et al.* who used biotin-requiring coryneform bacteria.[8] By using cross-feeding studies with *E. coli bio* mutants, they demonstrated that coryneform bacteria lack of the enzymes involved in the early steps of the pathway, encoded by the *bioF*, *bioC*, and *bioH* genes. Taken together, the above results indicate that biotin auxotrophic microorganisms lack the functional genes involved in the early steps of the biotin biosynthetic pathway.

To confirm whether biotin auxotrophy is due to a defect of the above genes (*bioW*, *bioF*, *bioA*, and *bioD* genes) in the *bio* operon of the OK2 strain, we attempted to repair those genes by homologous recombination with the whole *bio* operon of *B. subtilis* 168. Strain OK2 was transformed with amplified DNA containing the *bio* operon by using the primers BW-1 and BI-2 and *bio*$^+$ transformants were obtained on biotin-free medium. The nucleotide sequence analysis of five *bio*$^+$ transformants confirmed that all of the transformants contained the substituted *bio* operon (data not shown). To evaluate the biotin prototrophy, we examined the growth of these transformants on biotin-free medium. Although the *bio*$^+$ transformants grew well in both biotin-free medium and biotin-containing medium, the growth rate of this strain decreased gradually over repeated cultivations in spite of no alteration in the nucleotide sequences of the *bio* operon (data not shown). These results suggest that although *bio*$^+$ transformants have biotin autotrophy, they are unstable genetically. Although *B. subtilis* 168 synthesizes pimelic acid as a true intermediate in the

early steps of biotin biosynthesis,[3] the precursor of pimelic acid is still unknown as well as in the case of *B. sphaericus*.[5] Therefore, we conclude that the early steps to produce pimelic acid are genetically unstable in the OK2 strain as compared with the case of *B. subtilis* 168.

Acknowledgments

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